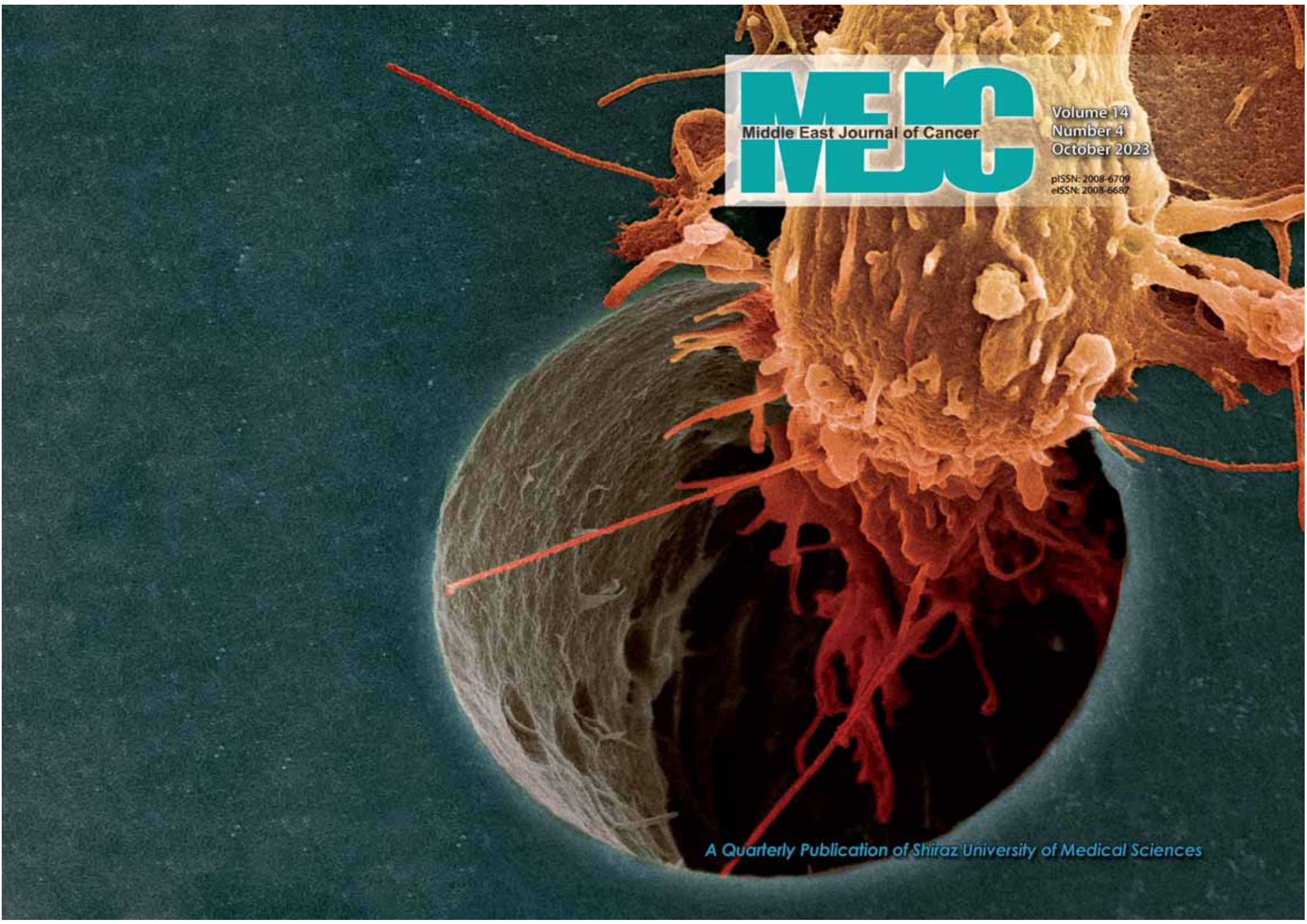

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References should be listed in the following style:

1. Handjani F, Delir S, Sodaifi M, Kumar PV. Lupus vulgaris following bacilli calmette-guerin vaccination. *Br J Dermatol* 2001;144(2):444-5.
2. Lippman, ME. Breast Cancer. In: Braunwald, E: Fauci, A: Kasper, DL: et al., editors. Harrison's principles of internal medicine, 15th ed. New York: McGraw-Hill, 2001:571-8.

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Table of Contents

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Middle East Journal of Cancer

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Review Article
Middle East Journal of Cancer; October 2023; 14(4): 471-480

Association between Non-alcoholic Fatty Liver Disease and Breast Cancer: A Systematic Review and Meta-analysis Study

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Abstract

Background: Breast cancer (BC) is the most prevalent neoplasm in females globally, with an increasing incidence trend almost in all regions. Previous studies have indicated that non-alcoholic fatty liver disease (NAFLD) may be an emerging risk factor for extrahepatic cancers, including BC. This systematic review and meta-analysis study aimed to determine the association between NAFLD and the development of BC.

Method: Data were systematically collected without time limitation until 21 April 2022, from the following electronic databases: PubMed, Scopus, Embase, Web of Science, and Google Scholar. The association between NAFLD and BC with odds ratio (OR) was calculated with a 95% confidence interval (CI) and presented via forest plots. Hazard ratios along with incidence rate ratios in the cohort studies transformed into OR.

Results: According to the preferred reporting items for systematic reviews and meta-analyses (PRISMA) and the inclusion criteria herein, 11 eligible studies were obtained from various countries. The pooled OR of NAFLD as a risk of developing BC, using a random-effects model, was estimated at 1.61 (95% CI: 1.30-2.00) (Q-value: 51.35, I2 = 80.52%, $P < 0.0001$). Multivariate meta-regression analysis showed that the publication year-, country-, detection method-, study design-, and body mass index-adjusted status did not cause heterogeneity. The Egger's regression ($P = 0.32$) and the symmetry in the funnel plot showed no publication bias in the studies.

Conclusion: The present research revealed that NAFLD had a significant association with BC, independent of traditional risk factors.

Keywords: Breast cancer, Non-alcoholic fatty liver disease, Systematic review, Association

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Introduction

Breast cancer (BC) is known to be the most prevalent neoplasm in females globally, with an increasing trend of incidence almost in all regions.[1,2] BC is the leading cause of cancer death in females. The mortality rates of this fatal cancer also increased in most regions, specifically in developing countries.[3]

BC accounts for about a quarter of all malignant deaths in postmenopausal women and, on a global scale, is the second leading cause of cancer deaths, after lung cancer, in the female population.[4]

In 2018, a total of 18.1 million new cases of cancer was reported, and 9.6 million cancer-related deaths occurred.[5]

The increase in BC incidence is due to the improvement of BC screening tools and the significant rise in exposure to various risk factors in the female population.[3,4]

The American Cancer Society recommends that women with an average risk of BC (relative risk of 2%–4%, those who have first-degree relatives with BC, CHEK2 mutation, age of above 35 for the first birth, proliferative breast disease, mammographic breast density)[6] should have a regular screening mammogram from the age of 45 years.[7]

Furthermore, hormonal or reproductive factors, such as late age to menopause,[8] young age at menarche, null parity, delayed pregnancy, and family history, are the known risk factors for BC.[4,7]

A meta-analysis study on women showed that obesity, alcohol consumption, and birth control pills as modifiable risk factors were associated with BC.[8]

Non-alcoholic fatty liver disease (NAFLD) is one of the most commonly reported chronic liver diseases globally, with an overall prevalence of 25.2% worldwide and 29.62% in Asia.[9] Although NAFLD prognosis is generally good, it ranges from hepatic steatosis (HS) to non-alcoholic

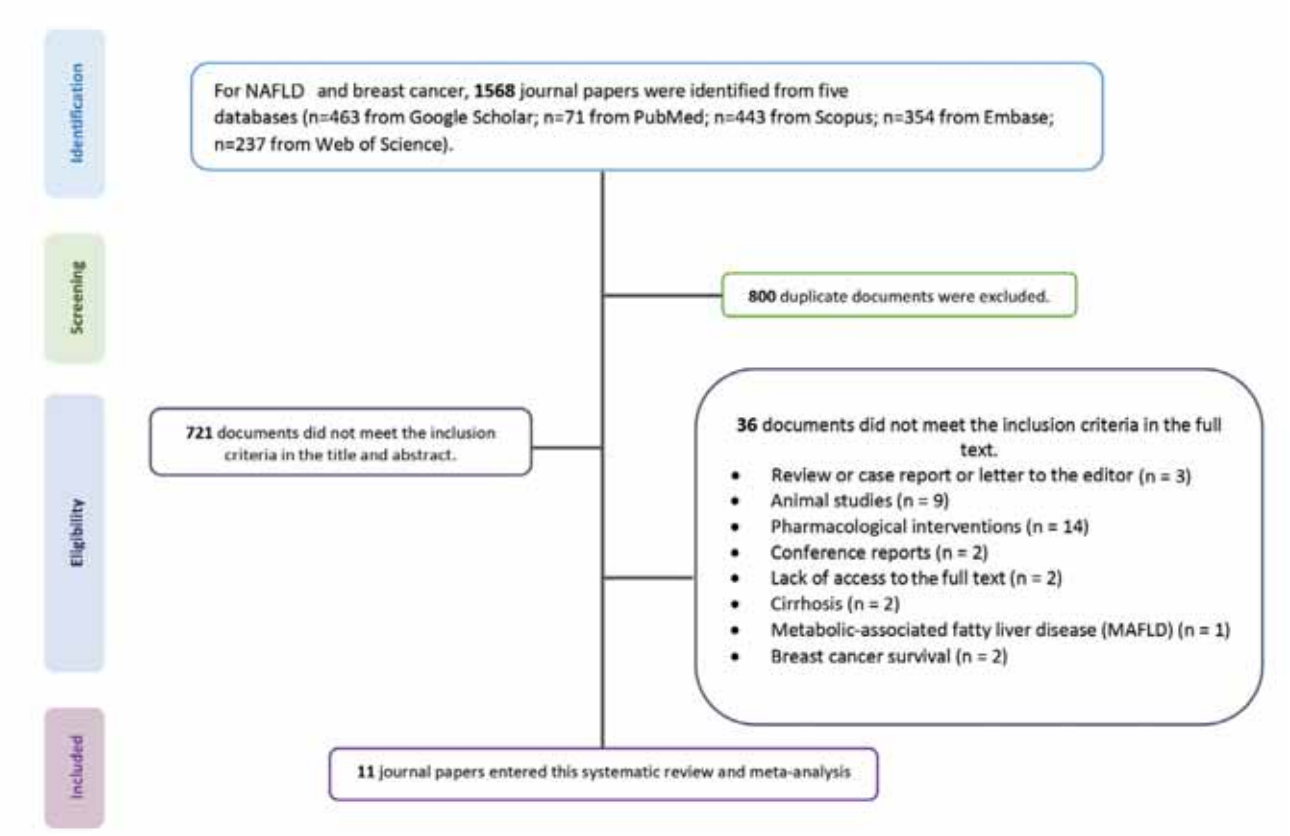

Figure 1. PRISMA flowchart presents the selection of the articles analyzed in this systematic review and meta-analysis.
n: Number; MAFLD: Metabolic-associated fatty liver disease; NAFLD: Non-alcoholic fatty liver disease

steatohepatitis (NASH) and cirrhosis.[7]

Some studies have suggested that NAFLD is a multisystem disease with extrahepatic complications,[10, 11] such as cardiovascular disease, chronic renal disease, decreased lung function, and extrahepatic malignancies.[7,12]

Malignancies are the second most common cause of death following cardiovascular disease in patients with NAFLD.[9, 13] Other studies have indicated that NAFLD may be an emerging risk factor for extrahepatic cancers, including BC.[7, 14]

To the best of our knowledge, no systematic review and meta-analysis independently have been published to estimate the linkage between NAFLD and BC. Therefore, this systematic review and meta-analysis paper aimed to determine the association between NAFLD and the development of BC.

Materials and Methods

This study was designed via the preferred reporting items for systematic reviews and meta-analyses (PRISMA).[15]

Bibliographic search strategy

The related studies with English language were identified from five English sources, namely PubMed, Scopus, Embase, Web of Science, and Google Scholar, without time limitation until 21 April 2022. The search was performed using the Medical Subject Heading (MeSH) terms as follows: (Breast Neoplasm) OR (Breast Tumors)) OR (Breast Tumor)) OR (BC)) OR (Mammary Cancer)) OR (Breast Malignant Neoplasm)) OR (Breast Malignant Tumors)) OR (Breast Carcinoma)) AND (NAFLD) OR (NAFLD)) OR (NAFLD) OR (NAFLD)) OR (Non-alcoholic Fatty Liver)) OR (NASH)) OR (NASH). In addition, the list of bibliography of all the selected articles or their citations were manually searched in Google Scholar to find other relevant articles. Figure 1 illustrates the study selection process in PRISMA flowchart.

Inclusion and exclusion criteria

After eliminating duplicates, the title and abstract of the related studies were screened. Subsequently, the full-text of the papers was reviewed by two authors independently to check the inclusion and exclusion criteria and assess the articles' quality. Contrasts of opinion between the reviewers were resolved by a third person alone and in consensus.

The inclusion criteria herein were as follows: 1) observational studies (case-control studies and cohort studies that investigated the association between NAFLD and BCs); 2) risk estimates, including odds ratio (OR), hazard ratio (HR), or incidence rate ratio (IRR), whose 95% confidence intervals (CI) were reported or could be calculated

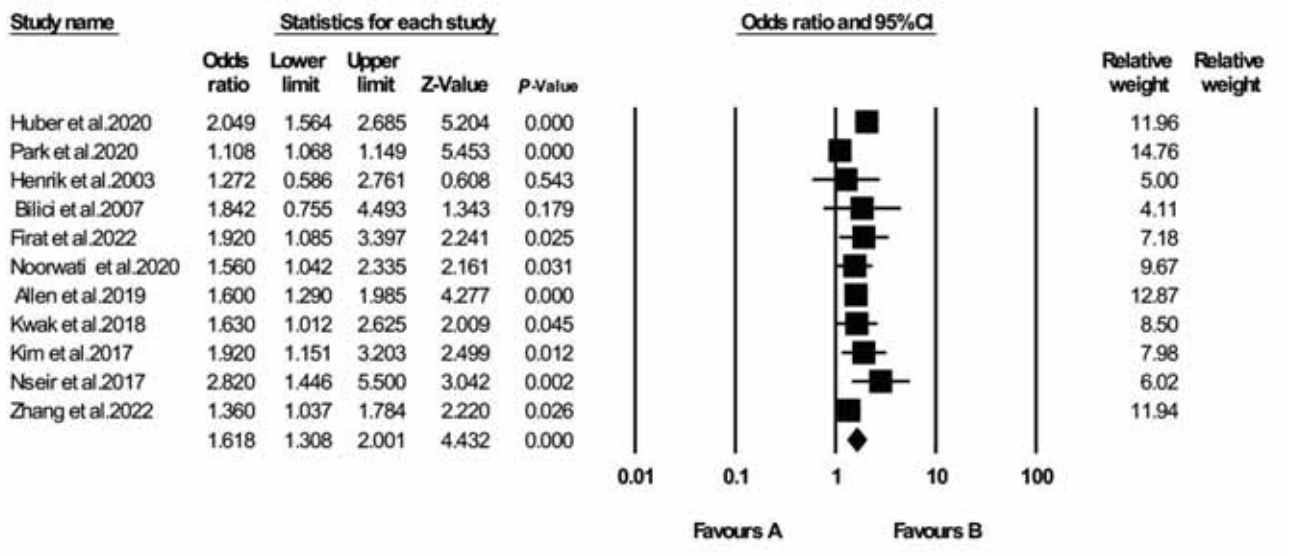

Figure 2. This figure shows the association between NAFLD and breast cancer.
NAFLD: Non-alcoholic fatty liver disease; CI: Confidence interval

Table1. Summary of the studies concerning the association between NAFLD and BC

Study	Country	Sample size	Age (years)	OR (95% CI)	NAFLD diagnosis	BC diagnosis	Study design	Adjusted confounding factors	QS
Fýrat et al., 2022[19]	Turkey	210	Control:54.5 ± 11.6 Case:52.4 ± 10.1	1.92 (1.08-303)	Hepatic ultrasonography	Mammography	Case-control	Age, BMI, prevalence of HT, DM, HL	4
Noorwati et al., 2020[21]	Indonesia	436	50	1.56 (1.04-2.33)	High-end ultrasound equipment.	Medical records	Case-control	-	4
Huber et al., 2020[20]	Germany	30324	58 ± 14	2.04 (1.56-2.68)	Medical record	Medical record	Cohort	HT, DM, dyslipidemia, obesity, BMI, age, sex, physician, index year, and CCI	5
Park et al., 2020[11]	Korea	7046153	49.08 ± 14.49	1.10 (1.01-1.14)	FLI	Medical record	Cohort	Age, smoking status, drinking, regular exercise, DM, and BMI	8
Allen et al., 2019[23]	USA	10204	54	1.60 (1.30-2.0)	Medical record.	Medical record	Cohort	Age and sex	8
Kwak et al., 2018[7]	Korea	444	Control:51.6 ± 9.3 Case: 51.7 ± 9.3	1.63 (1.01-2.62)	Hepatic ultrasonography	Mammography	Case-control	Menstrual and reproductive factors, age, and BMI	7
Kim et al., 2017[9]	Korea	11981	53.2±9.5	1.92 (1.15-3.20)	Hepatic ultrasonography	Pathological	Cohort	Age and sex	8
Nseir et al., 2017[24]	Occupied Palestinian Territory	146	Case: 54.8 ± 12 Control: 57.5 ± 9.6	2.82 (1.44-5.50)	Abdominal CT examination	Mammographic	Case-control	Age and BMI	9
Bilici et al., 2007[17]	Turkey	80	Case:47.5 ± 11.9 Control:43.4 ± 6.0	1.84 (0.75-4.49)	Hepatic ultrasonography	Medical record	Case-control	Age	5
Henrik et al., 2003[22]	Denmark	840	56	1.27 (0.58-2.76)	Medical record	Pathological	Cohort	-	5
Hong et al., 2022[5]	China	1976	Case: 50.0 ± 10.9 Control:50.6 ± 10.9	1.36 (1.04-1.79)	Hepatic ultrasonography	Ultrasonography	Case-control	-	6

QS: Quality study; NAFLD: Non-alcoholic fatty liver disease; BC: Breast cancer; CCI: Charlson comorbidity index; BMI: Body mass index; CT: Computerized tomography; OR: Odds ratio; FLI: Fatty liver index; CI: Confidence interval; DM: Diabetes mellitus; HT: Hypertension; HL: Hyperlipidemia;"-": not applicable

using the data reported in the articles; 3) studies with full-text access published in English.

On the other hand, inconsistency in data, the use of inappropriate statistical methods, uncertainty of sampling method, duplicate articles, review articles and meta-analysis, letter to editor, short reports, case reports, case series, cross-sectional studies, conference reports, animal studies, and papers that did not have enough data to calculate the OR were the excluded from the current research.

It should be mentioned that in this study, NAFLD was defined by histopathologic tests, imaging, or ICD-10 codes, demonstrating HS, or medical record. BC was defined based on pathology tests, mammography, and medical records.

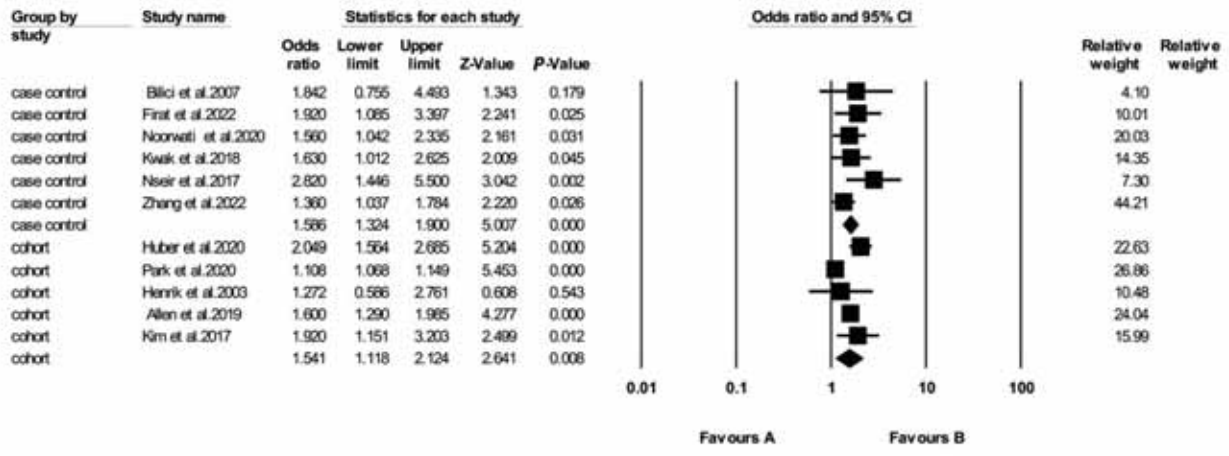

Figure 3. Subgroup meta-analysis of the association between NAFLD and breast cancer based on the study design.
NAFLD: Non-alcoholic fatty liver disease; CI: Confidence interval

Data collection

An Excel data extraction form was used for collecting the following data from eligible studies: the first author, year of publication, country of study, sample size, NAFLD diagnosis, BC diagnosis, study design, and adjusted confounding factors (Table 1).

Quality assessment (risk of bias)

The quality of the included studies was evaluated based on the Newcastle–Ottawa Scale (NOS). According to the NOS assessment score, the quality of a study was considered good (6<), moderate (3-5), and low (<3). Therefore, the studies with acceptable (moderate and good) quality were eligible for meta-analysis.[16]

Statistical analysis

The association between NAFLD and BC with OR was calculated with a 95% CI and presented via forest plots. In this plot, OR greater than one indicates a risk factor, and OR less than one shows a protective effect. HRs and IRRs in the cohort studies transformed into OR. The expected heterogeneity among the studies was evaluated with statistical methods, Cochran's Q test, and the I^2 index. Egger's regression was utilized for publication bias assessment.

A fixed-effect model was used when there was no literature heterogeneity. Otherwise, we employed the random effect model. Through the use of the multivariable meta-regression model and subgroups analysis, the effects of probable factors in heterogeneity were investigated. The meta-analysis was conducted with the trial version of Comprehensive Meta-Analysis software vs. 3.

Results

Search results and eligibility of the studies

In this systematic review, 1568 articles were found by searching the entire databases and considering the inclusion criteria. Afterwards, we removed 800 articles due to duplication, as well as 721 papers due to non-compliance with the inclusion criteria in the title and abstract.

However, 36 articles were excluded according to the exclusion criteria after reading the full-text of articles, including: review or case report or letter to the editor (n = 3), animal studies (n = 9), pharmacological interventions (n = 14), conference reports (n = 2), lack of access to full-text (n = 2), cirrhosis studies (n = 2), metabolic-associated fatty liver disease (MAFLD) (n = 1), BC survival (n = 2), and risk factors for NAFLD (n = 1).

Finally, 11 studies met the evaluation criteria, which entered this study (Figure 1).

Characteristics of the eligible studies

The total eligible studies contained 11 journal papers with 7,102,785 as the sample size. The smallest sample size belonged to a case-control study in Turkey[17] with 80 subjects and the largest sample size to a cohort study in Korea[18] with a sample size of 7,046,153.

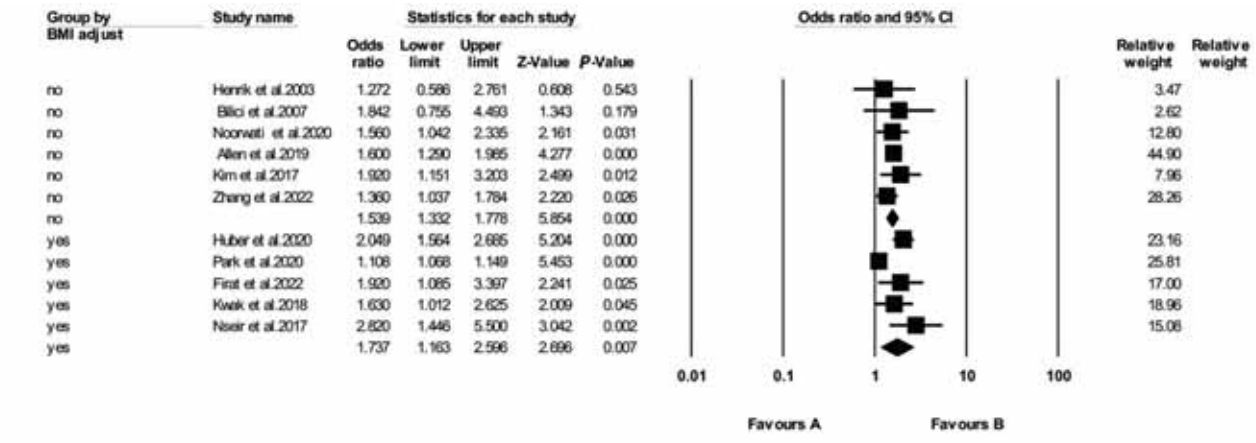

Figure 4. Subgroup meta-analysis of the association between NAFLD and breast cancer adjusted based on BMI variable.
NAFLD: Non-alcoholic fatty liver disease; BMI: Body mass index; CI: Confidence interval

Table 2. Result of multivariate meta-regression model for detecting probable sources of heterogeneity

The probable source of heterogeneity	Multivariable	
	Coefficient (95%CI)	*P*-value
Year	0.003(-0.054-0.060)	0.91
Country	-0.08 (-1.05-0.87)	0.69
Design of the study	-0.18(-0.69-0.32)	0.47
Detection method of breast cancer	-0.38 (-1.59-0.82)	0.78
Status of adjusted BMI	-0.01 (-0.54-0.57)	0.96

BMI: Body mass index; CI: Confidence interval

Based on geographical regions, three studies were performed in Korea,[7,9,18] two in Turkey,[17,19] one in Germany,[20] and one in Indonesia,[21] Denmark,[22] the USA,[23] Occupied Palestinian Territory,[24] and China[5] (Table 1).

In addition, there were five cohort and six case-control studies. The method of BC diagnosis was mammography (n = 3), pathology (n = 2), ultrasonography (USG) (n = 1), and the use of medical records (n = 5) (Table 1).

According to the NOS quality assessment, no studies scored as low quality, five had medium quality, and the other six was revealed to have good quality (Table 1).

Association between NAFLD and BC

A total of 7,102,785 women, including 62,886 women with BC, were studied. The pooled OR of BC was analyzed based on 11 studies in order to examine the association between NAFLD and BC risk. Utilizing a random-effects meta-analysis, the overall OR of NAFLD, as a risk of developing BC, was estimated at 1.61 (95% CI: 1.30-2.00) (Q-value: 51.35, I2 = 80.52%, $P < 0.0001$) (Figure 2).

Multivariable meta-regression analysis showed that the publication year-, country-, BC detection method-, study design-, and body mass index (BMI)-adjusted status did not represent heterogeneity (Table 2).

The results of subgroup analysis displayed that the pooled OR of BC in the case-control studies was 1.58 (95% CI: 1.32-1.90), which was 1.54 (95% CI: 1.11-2.12) in the cohort studies (Figure 3).

Additionally, the pooled OR of BC in the studies was revealed, where the BMI variable was adjusted at 1.73 (95% CI: 1.16-2.59) and not adjusted study at 1.53 (95% CI: 1.33-1.77) (Figure 4).

Publication bias

The funnel plot and Egger's test were used for

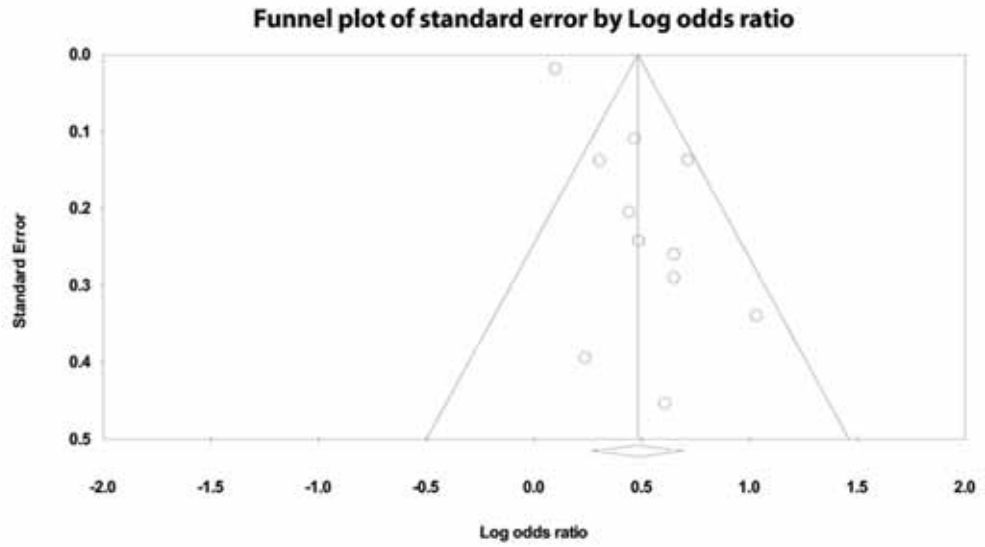

Figure 5. This figure shows the funnel plot with pseudo 95% confidence limits for detection of publication bias among the included studies.
Log: Logarithm

assessing the presence of publication bias. The result of Egger's regression (P = 0.32) and the symmetry in funnel plot interpretation indicated no publication bias in studies, as displayed in figure 5.

Discussion

This systematic review was designed in order to evaluate the link between NAFLD and BC. In this study, we analyzed a total of 7,102,785 subjects, including 62,886 women with BC. The pooled OR was analyzed based on 11 studies to examine the association between NAFLD and BC risk. The overall OR of NAFLD, as a risk of developing BC, was estimated at 1.61 (95% CI: 1.30-2.00).

Early diagnosis and accurate therapy are critical for BC. A number of predictive BC risk models have been developed, but none of the research considered NAFLD.[25, 26] Over the past years, the association between NAFLD and BC has attracted a great deal of scientific attention.

Certain papers have indicated that BC is a common extrahepatic complication of NAFLD.[14, 27] It is known that NAFLD causes liver, heart, and kidney diseases.[12] Furthermore, numerous studies on the risk of extrahepatic malignancies have shown a link between NAFLD and certain types of cancer.[11, 14, 19]

The present study shed light on the significant association between NAFLD and BC, so that confirmed NAFLD as an independent risk factor for women with BC. These results are consistent with those reported in previous studies, demonstrating an association between NAFLD and BC.[7, 14, 21, 24] Accordingly, NAFLD is linked to BC, regardless of the known risk factors.

A case-control study showed an association between NAFLD and BC in Occupied Palestinian Territory;[24] however, the sample size was small, at just 73 cases. Furthermore, BC incidence and the outcomes vary according to ethnic background.[7, 28]

Kwak et al. found a statistically significant difference in NAFLD patients with non-obese BC and a control group.[7] Lee et al. also demonstrated that NAFLD is a predictor for BC and a prognostic factor for its recurrence.[29] In a Korean study that included patients with non-cirrhotic NAFLD, a 1.9-fold greater incidence of BC was observed in women.[30] Other cohort studies also revealed a relationship between NAFLD and BC incidence.[9, 27]

Some possible mechanisms can explain the relationship between BC and NAFLD.[7, 31] Primarily, NAFLD is closely associated with increased pro-inflammatory cytokine levels, such as tumor necrosis factor alpha and interleukin-6, and decreased adiponectin levels,[7, 27] promoting cancer through tumor cell proliferation, anti-apoptotic effects, and angiogenesis.[7, 32] Secondly, NAFLD plays a significant role in developing systemic insulin resistance;[32] insulin can bind to insulin-type I growth factor receptor (IGF-1) expressed on breast cells, and downstream signaling pathways stimulate the proliferation of BC cells.[33] In addition, hyperinsulinemia can increase hepatic synthesis of IGF-I, while decreasing liver expression of IGF-1 binding proteins, resulting in elevated levels of free IGF-I.[34] These changes in NAFLD may lead to BC development.[17, 35]

The diagnosis of NAFLD can be generally confirmed through imaging studies, and the disease can be staged through liver biopsy.[9] In practice, it is difficult to perform a liver biopsy for routine screening due to the invasive and non-economic nature of the procedure.[21, 36] The essential imaging examinations for the diagnosis of liver steatosis include ultrasound computerized tomography and magnetic resonance imaging.[24] Ultrasound is used extensively in clinical practice and health screening to detect liver fat infiltration.[21, 37] However, ultrasound is not sufficiently sensitive for slight steatosis detection and cannot quantify the severity of steatosis in hepatocytes.[24] USG at 60%-70% sensitivity is commonly used in clinical practice.[38, 39] USG sensitivity can arise once two radiologists are present.[17]

Our research also revealed the pooled OR of BC in studies where the BMI variable was adjusted at 1.73 and not adjusted as a confounder variable was 1.53. That mentioned, when the

effect of BMI as a confounding factor is not controlled, the association between non-alcoholic fatty liver and BC is weaker. Still, when its effect is controlled as a confounding factor, the association between fatty liver and BC becomes stronger.

Noorwati Sutandyo et al. demonstrated that HS plays a more critical role as a risk factor in BC occurrence compared with anthropometric BMI.[21] Even though fatty liver is associated with increased BMI, the risk of BC may not be linked to general obesity. The logical explanation for this conclusion is thought to be another factor(s) in the pathogenesis of fatty liver disease, which is also responsible for developing BC.

We herein demonstrated that NAFLD could be a significant intermediate biomarker of BC risk. These results could be put in use as a source of hypotheses for future studies on the biological mechanisms underlying this relationship, considering NAFLD as the main predictor or as a mediator variable in the causal pathway of BC development.

The strengths of this study include the comprehensive search strategy in five international databases, the large total sample size, the stringent methodology, and meta-analysis subgroups, including study design- and BMI-adjusted status.

This study had certain limitations that should be considered. To begin with, we did not assess the known risk factors herein, such as family history of BC, diabetes, breastfeeding, tobacco use, hormone replacement treatment, and a history of benign breast disease (such as atypical hyperplasia). Furthermore, in all the studies in our systematic review NAFLD diagnosis was made using USG, a non-invasive imaging method, rather than biopsy. It could be suggested that future research use magnetic resonance imaging, a non-invasive and susceptible test.

Conclusion

In conclusion, the current study revealed a significant association between NAFLD and BC, independent of traditional risk factors. Further research is needed to determine which BC subtype is most associated with NAFLD and determine BC screening recommendations for women with NAFLD. Moreover, these results warrant further research to assess the mechanism of BC in women in association with NAFLD. Our findings provide a platform for other mechanistic studies of NAFLD as a hidden vector or interim biomarker of cancer risk in obesity.

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Availability of Data and Materials

The research data used to support the findings of this study are available from the corresponding author of this work upon request.

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Ethics Approval

The authors of this study followed the ethical principles of systematic reviews, including guidance on authorship, avoiding redundant (duplicate) publication, avoiding plagiarism, transparency, and ensuring accuracy with no potential complications. This review was not registered.

Conflict of Interest

None declared.

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Review Article
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The Emerging Role of Chitosan-based Polymeric Nanoparticles in the Diagnosis and Treatment of Gynecological Cancers

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Abstract

Breast and gynecological cancers are the most common malignancies in females. Early-stage detection and treatment could significantly reduce the mortality rate in patients. However, common treatments such as chemotherapy and radiotherapy fail after a while and lead to recurrence and drug resistance in cancer cells. The recent use of nanotechnology has enabled the development of novel approaches for diagnosing and treating oncological diseases. Chitosan-based polymer nanoparticles (CHPNPs) with unique properties such as non-toxicity, biocompatibility, and anti-carcinogenic effects are promising tools for the clinical development of targeted delivery systems. So far, various methods have been applied to use these nanoparticles in the diagnosis and treatment of various cancers. Identifying the most practical methods is one of the most important challenges in achieving effective treatments. A review of these studies can provide better horizons to realize effective treatment. In this review, we evaluate and discuss the use of CHPNPs from published literature to assess diagnostic and therapeutic strategies in breast and gynecological cancers, including ovarian and uterine neoplasms, as well as their advantages and challenges.

Keywords: Chitosan nanoparticles, Nanotechnology, Breast neoplasms, Ovarian neoplasms, Uterine neoplasms

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Introduction

Nowadays, nanotechnology is a topic of great interest in research and medicine.[1] The study of sub-micron particles is becoming increasingly important due to their potential ability to transport drugs and target specific systems. Nanoparticle-based drugs have higher efficiency and can overcome the typical challenges of

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regular drug distribution systems.[2] Nanomedicine has the potential to facilitate precision medicines, improve therapeutic results, and reduce adverse side-effects.[3] Accurate and targeted delivery of pharmaceuticals to specific cells and tissues make them more efficacious and significantly improve outcomes.[4] Therapeutic compounds encapsulated in nanoparticles can endure longer in the bloodstream, are not easily hydrolyzed, have increased efficacy, and have greater opportunities to cross cell membranes and be taken up by cells.[5] Specific cells can be targeted by attaching special ligands to the surface of the nanodrug.[6] Among the types of nanocarriers used to transfer drug compounds, some have been studied more extensively due to their special properties and favorable results. Nanoparticles have different types, such as Lipid-based NPs, Inorganic NPs, and Polymeric NPs (Figure 1).[7, 8]

Polymeric nanoparticles are solid colloidal, rod-like, or spherical materials made of natural or synthetic materials, and by creating different structures, they display various features.[9, 10] Due to their biocompatibility and simple formulation, this nanoparticle can be a suitable means of transfer.[11] There are several methods for producing polymeric nanoparticles, each of which creates a specific product. By knowing the characteristics of each product, compatible materials (hydrophilic and hydrophobic) can be best embedded in nanoparticles and facilitate their transfer to the target cells, making polymeric NPs suitable for co-delivery uses. Drugs are dissolved, trapped, encapsulated, or absorbed in the polymer matrix composition. (Table 1).[12] Chitosan is one of the most widely used polymeric nanoparticles. It was first created in 1859 from a chitin biopolymer.[13] Chitosan is a de-acetylated form of chitin. Chitin is a natural biopolymer found in the exoskeleton of marine crustaceans such as lobsters, crabs, and the cell wall of fungi.[14, 15] Chitosan has several desirable attributes such as nontoxicity, biocompatibility, anti-carcinogenic, fungistatic, low immunogenicity, and bacteriostatic. It can interrupt

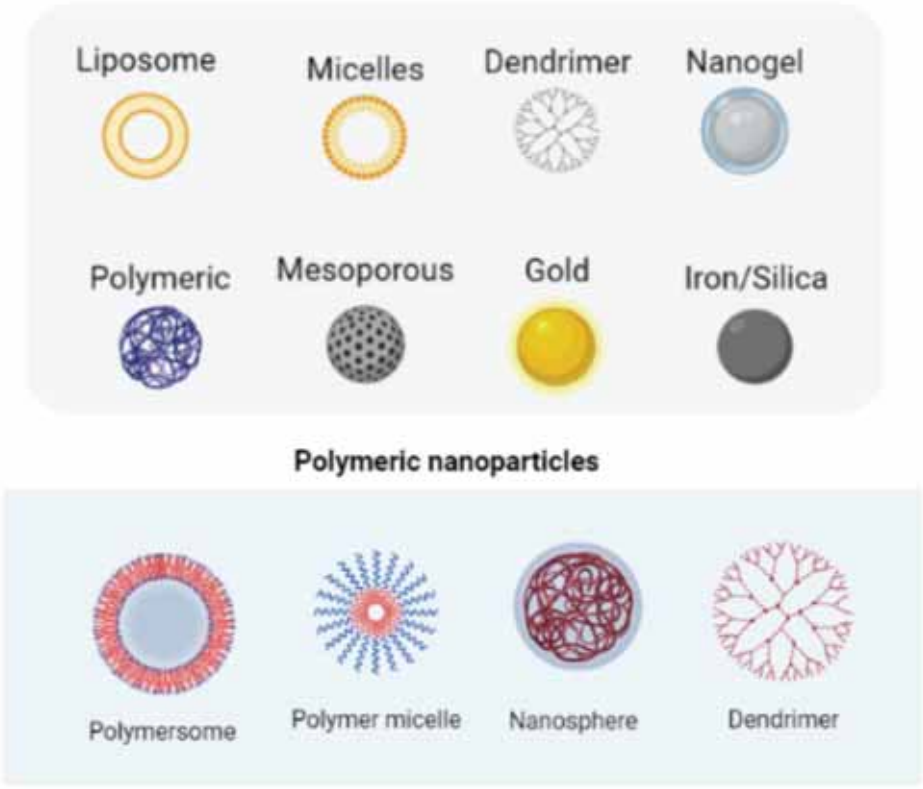

Figure 1. This figure shows the different types of nanoparticles and polymeric nanoparticles.

intercellular connections, making them more permeable.[16, 17] Chitosan is a hydrophilic polymer with one amine group and two hydroxyl groups. It can be easily attached to different functional groups by having an active amine group. The amine group causes its solubility in acidic solutions by creating a poly-electrolytic property in it. The amine groups affect a large variety of chitosan pharmaceutical and biomedical features, including mucoadhesion, penetration increment, and in situ occlusion.[5, 18] Chitosan is slightly hydrophobic due to the presence of the N-acetyl group (Figure 2).[19]

Chitosan nanoparticles preparation methods

There are several ways to create chitosan nanoparticles, and the method of preparation is a key step in ensuring that the particulates behave as intended,[20, 21] playing a vital role in achieving the desired properties. Ionic gelation, emulsion cross-linking, spray-drying, emulsion droplet coalescence method, nanoprecipitation, reverse micellisation method, desolvation/simple coacervation / phase separation, modified ionic gelation with radical polymerization, and emulsion solvent diffusion are some prevalent methods.[12, 22] It should be noted that certain characteristics of chitosan-mediated drug delivery systems, such as particle size, toxicity, different interactions of chitosan nanoformulation and drugs, thermal and chemical stability, and kinetics, strongly depend on the selected preparation methods.[23]

The surface of chitosan-based polymeric nanoparticles can be easily altered to target specific tissues. Thus, the use of these nanoparticles as a cell-specific targeting system can prevent non-specific interactions, side-effects, and toxicities of drugs.[24] In addition, specific biomarkers can be conjugated to chitosan for more precise detection and imaging of malignancies.[25, 26] Nevertheless, it is worth noting that the challenges of polymeric nanoparticle application are an increased risk of particle accumulation and low toxicity in some cases.[27] Currently, only a limited number of polymeric nanomedicines are FDA approved and applied in the clinic, but different types of polymeric nanocarriers are now undergoing investigation in many clinical trials.[28]

Table 1. Method of preparation of Chitosan-based polymeric nanoparticles

Method of preparation of CSNPs
Ionic gelation/polyelectrolyte complexation
Emulsion droplet coalescence
Emulsion solvent diffusion
Reverse micellisation
Desolvation
Modified ionic gelation with radical polymerisation
Emulsification cross-linking
Nanoprecipitation
Spray-drying

CSNPs: Chitosan-based polymeric nanoparticles

Gynecological cancers

Female-specific malignancies (FSMs) such as breast cancer (BC), ovarian cancer (OC), and uterine cancer (cervical cancer and endometrial cancer) have a profound effect on the health of women worldwide and play a significant role in the global cancer burden.[29, 30] Each type of female-specific cancer has unique epidemiological and genetic risk factors, symptoms, prognosis, and response to therapy, and they cannot be easily diagnosed or treated.[31] The prevalence of these

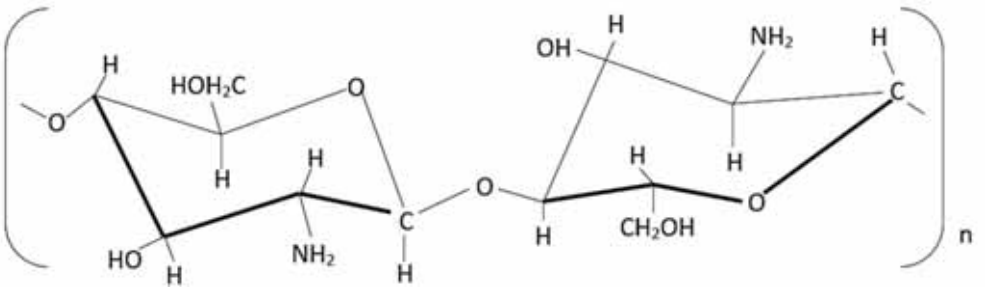

Figure 2. This figure depicts the chitosan chemical structure.

Table 2. Biological ligand in breast and gynecological cancer

Biomarker	Technology used for discovery	Type	Type of cancer
RS/DJ-1	Serum profiling	Serum protein	BC
CA125			EC/OC
CA15-3			BC/OC
Ca19-9			EC/OC
CA27-29			BC
Gal-3			OC
CA72.4			OC
HER-20			BC
HE4			EC/OC
P53	Humeral response	autoantibody	BC
p16			VC
CYFRA21-1			UC
HSP60			BC
SCC-Ag			CC
HSP90			BC
MUC1			BC
α-2-HS-Glycoprotein	Nipple aspirate fluid profiling	Ductal protein	BC
Lipophilin B			BC
β-Globin			BC
Hemopexin			BC
Vitamin D-binding			BC
protein			BC

BC: Breast cance; EC: Endometrial cancer; OC: Ovarian cancer; UC: Uterine cancer; VC: Vulvar cancer; CC: Cervical cancer

cancers is such that, by 2020, it was estimated that more than 3 million new patients and more than 1 million cancer deaths worldwide. Most gynecological diseases are associated with infertility, which causes chronic stress and various psychological problems, adversely affecting the normal life-style of families and burdening the health of society. With the physical and mental comfort of fertile women, the family can be a more suitable platform for the growth and education of children, and healthier children will be delivered to society.[32-34] Innovating new

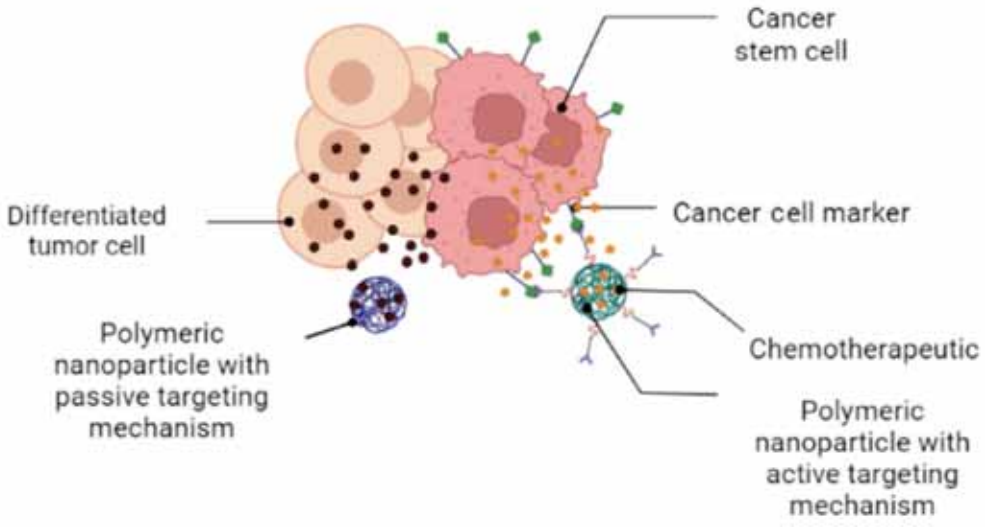

Figure 3. In this drug delivery system, drug targeting is performed by identifying the target agent that binds to the receptors on the target cells at the level of the drug delivery system. The target group includes bioadhesive nonionic surfactants,

Table 3. Uterine serous and clear cell carcinomas are rare but more metastatic and related to a poor prognosis. Uterine sarcomas contain 2 to 5% of uterine malignancies and emerge from the myometrium or other mesenchymal structures

Gynecologic oncology group classifications of uterine sarcomas
Non-epithelial tumors
Endometrial stromal tumors
Stromal nodule (benign)
Endometrial stromal sarcoma
Undifferentiated endometrial sarcoma
Leiomyosarcoma
Myxoid
Smooth muscle tumors of uncertain malignant potential
Mixed endometrial stromal and smooth-muscle tumors
Mixed epithelial-non-epithelial tumors
Adenosarcoma
Homologous
Heterologous
Adenosarcoma with high-grade overgrowth

methods of diagnosis and treatments that can potentially improve the therapeutic outcome for cancer patients is much needed. In this review, we focus on current documents for the efficacy of CHPNPs and their new applications in the diagnosis and treatment of female-specific cancers.

Biological ligands in breast and gynecological cancer

Lately, much attention has been paid to the identification of biological markers for use as indicators of disease activity, as well as prognostic factors and predictors of survival, recurrence, and treatment response in female patients. There are various markers for breast and gynecological cancer diagnosis. DNA biomarkers provide information on the process of tumor formation, but they are related to poor early diagnosis because of low concentrations of cancer markers. Serum tumor biomarker assessment is a strategy to evaluate tumor presence, recurrence, or response to treatment in gynecological cancer patients.[35, 36] Protein biomarkers are the main index of BC, which can be arranged as two main markers. Predictive protein markers provide information on a certain treatment intervention, while prognostic protein markers suggest general information on the issues (Table 2).[37]

OC

OC is the 8th leading cause of cancer mortality in women.[38] Due to the lack of clinical symptoms, it is often diagnosed too late in advanced stages (stage III and IV). OC includes four stages: stage 1 is limited to one or both ovaries, stage 2 spreads to pelvic viscera (bladder, uterus, ovarian tubes, or rectum), stage 3 extends to the abdominal lining, abdomen, and lymph nodes, and finally, stage 4 spreads to abdominal organs (liver, intestine, and spleen), and even the lungs in the thoracic cage may be involved. Tumor cells' metastasis into the peritoneal cavity significantly reduces the chance of treatment.[39-41]

Diagnosis

There are some trials for early disease diagnosis, such as clinical histories, physical examination, tissue biopsy, ultrasound assessment, positron emission tomography (PET), and computed tomography (CT) scan. An efficient method for early detection of OC is to evaluate cancer antigen-125 (CA-125) serum protein that rises in 80% of women with OC. In other words, an increase in serum CA-125 is a sign of treatment failure.[42, 43] Circulating tumor DNA evaluation is a novel specific technique that is being used recently that can precisely diagnose tumor cells and malignancy.[44] Lysophosphatidic acid is another option for assessment in women with benign gynecologic diseases.[45]

Treatment

Surgery, radiation therapy, and chemotherapy have additional benefits on survival.[46] Current shreds of evidence indicate that OC cells are relatively resistant to classical chemotherapy, and there has been only an approximate improvement

Table 4. Endometrioid adenocarcinoma arises from the endometrium and is the most common pathologic subtype (95% of cases)

Classifications of endometrial carcinomas
Endometrial adenocarcinoma
Adenocarcinoma with squamous differentiation
Villoglandular
Secretory
Ciliated cell
Uterine serous carcinoma
Clear cell carcinoma
Mucinous carcinoma
Carcinosarcoma
Squamous cell carcinoma
Mixed adenocarcinoma and other rare variants

in the overall survival of OC patients. [47] Overall, in these patients, most treatment strategies have led to a high rate of relapse and poor outcomes that required more endeavors to advance beneficial therapeutic methods.[48]

Nanotechnology has an important impression on the diagnosis and treatment of OC.[49] In chemotherapy, the manufactured nanosystem must have significant drug loading capacity, drug dissolving capacity in the inner core, and selective aggregation in target tumor tissue through the effects of permeability and retention. In addition, the development of specific ligand-functionalized nanoforms will enable special targeting of ovarian tumors and ultimately increase therapeutic potential compared with non-functional counterparts.[50-52]

Chitosan-based nanostructured units have been highly used for effective delivery of biomolecules and macromolecules, including nutrients, proteins, vitamins, phenolic, and hydrophobic drugs in diverse biological systems.[53] S´anchez-Ramírez et al. designed a biocompatible and biodegradable nanocarrier system based on chitosan (lactic-co-glycolic) (PLGA) synthesized CP-ICG NPs for competitive trapping of photoactive drugs and chemotherapy (CP), and its potential for anti-cancer activity was evaluated. These nanoparticles showed cytotoxic and antitumor effects on the SKOV3 OC cell line after irradiating the cells with an 800 nm laser.[54]

Recently, curcumin loaded on poly lactic-hemaglycolic acid (PLGA), a biodegradable nanoparticle (CUR-NP), was tested against SKOV3 human ovarian adenocarcinoma cells by photodynamic therapy. Increased stability was observed compared with free curcumin and also showed strong apoptosis.[55] Pakchin et al. has developed an immune-based electrochemical nanosensor to identify CA-125. This nanosensor was designed based on polyamidoamine/gold nanoparticles and 3D reduced graphene oxide/multiwall carbon nanotubes nanosensor. In order to increase the conductivity and the number of antibodies (Abs) immobilized on the electrode outward, Polyamidoamine/gold nanoparticles (PAMAM/AuNPs) were used. Toluidine blue and antibody appended to O-succinyl-chitosan-magnetic nanoparticles (Suc-CS@MNPs) as a detector. They improved the insignificant solubility of chitosan with succinic anhydride, applying a novel rectification technique. The reliability of the constructed nanosensor in detecting CA-125 was verified by standard addition recovery method.[56]

OC frequently spreads to peritoneum and causes enormous aggregation of fluid (ascites). By isolating and analyzing cancer cells in ascites, unique and valuable information would be yielded. M. Castro et al. designed an ascites-specific microfluidic chip (ATC chip) that extracts ATCs from their profoundly inflammatory microenvironment. It's a simple and rapid ATC profiling approach that has the potential to expand the reach of point-of-care strategies and lead therapeutic clinical trials for OC.[57] Jia Xu et al. encapsulated hydroxyapatite nanoparticles and marizomib with chitosan to increase marizomib's

Table 5. Application of chitosan-based nanoparticles in the drug delivery system in preclinical and clinical studies

A substance used in integration with chitosan nanoparticles	Research findings	Reference
Preclinical research		
Curcumin	Increased Curcumin's anticancer activity against colon and breast cancer cells	(139)
Insulin	Decreased glycaemia was observed in diabetic rats	(140)
Marizomib	Increased absorption by cancer cells, induced apoptosis, and destroyed ovarian A2780 cancer cells	(141)
Raloxifene	Induced more apoptosis in breast cancer cells	(91)
Theophylline	Anti-inflammatory effects were noticeably enhanced	(142)
Paclitaxel	Significant inhibition of tumor progression and long survival	(136)
Clinical research		
Doxorubicin	Decreased Doxorubicin toxicity and tumor growth rate	(143)
Morphine	Improved morphine pain relievers	(144)

(Salinosporamide A. is an anticancer agent) efficacy and bioavailability. The created nanoparticles were efficaciously absorbed by cancer cells, induced apoptosis, and destroyed ovarian A2780 cancer cells.[58]

BC

BC is the leading cause of cancer death among women aged 20 to 59. In 2021, an estimated[59] 281,550 new cases of ductal carcinoma in situ (DCIS) of the female breast were reported. The incidence of BC continues to rise at about 0.5% per year.[60] BC can be categorized according to the molecular subtypes as luminal (A&B), human epidermal growth factor receptor 2 (HER2), and estrogen (ER) / progesterone receptor (PR) positive and triple negative.[61] Nearly 70% of all reported cases among all recognized subtypes are ER/PR positive subtypes.[62] Approximately 20% of BCs do not express HER2, ER, and PR, which is known as triple-negative (TN), and is basal-like (about 75%) and has an aggressive phenotype with a higher rate of metastasis.[63] The main cause of BC death has been reported to be the result of possible metastasis to distant organs such as the liver, lungs, lymph nodes, bones, and brain.[64]

Diagnosis

The most prevalent histopathology of BC is invasive ductal carcinoma (50%-75% of patients), followed by invasive lobular cancer (5%-15% of patients).[65] Identifying cancer cells in the early stages is the key to a better prognosis. The initial diagnosis involves self and clinical examination and radiographic scans (mammography, magnetic resonance imaging, ultrasound, CT, PET, microwave imaging) followed by invasive biopsy for the histological confirmation of invasive disease.[66-69] BC can also be diagnosed by biomarker-based methods such as radioimmunoassay, immunohistochemistry, enzyme-linked immunosorbent assay (ELISA), and fluoroimmunoassay. Some biomarkers are shown in table 2. Another new method for sensitive detection of cancer cells is optical biosensors, including fiber optics, fluorescence, resonant mirror sensors, interferometry, and surface plasmon resonance, which have been developed to detect target cancer markers.[70, 71]

Treatment

To increase the survival rate in cancer patients, the development of effective therapies against metastatic BC remains an important priority. The main purposes of treatment for non-metastatic BC, are to eliminate the tumor from the breast and associated lymph nodes to prevent metastatic

recurrence.[72-74] Routine treatment for non-invasive BC consists of lumpectomy, mastectomy, sampling, or removal of axillary lymph nodes, with consideration of postoperative radiation. Depending on the cancer subtype, systemic treatment (endocrine therapy for all HR+, trastuzumab-based ERBB2-directed antibody therapy plus chemotherapy for all ERBB2+ tumors, and chemotherapy alone for triple-negative BC is also applied.

For invasive BC, therapeutic targets are to increase life expectancy and relieve symptoms. Presently, invasive BC is incurable in almost all patients. The same basic categories of systemic therapy are used in invasive BC.[75-79]

Among all kinds of treatments, chemotherapy is commonly used for treating BC. Most cancer cells can be eliminated by efficient chemotherapy throughout the body. There are some approved anticancer medications such as tamoxifen, taxanes, paclitaxel, docetaxel, doxorubicin, raloxifene, and methotrexate used for the treatment of BC.[80, 81] However, the low bioavailability and poor aqueous solubility of these drugs have led to reduced treatment efficiency.

Two main strategies for better therapeutic efficacy and reducing chemotherapy side-effects are tumor-targeted delivery and managed release of these medications through nanoparticles. Drug-loaded nanoparticles compared to conventional chemotherapy drugs are considered a favorable tool for cancer treatments because of their high loading capacity, stability, specificity, tolerability, and reduced toxicity. The delivery system is designed to keep the therapeutic intact until it arrives at the desired location without any changes. Nanoparticles can actively and passively deliver anticancer drugs to cancerous tumors during treatments (Figure 3). Among different kinds of nanoparticles, polymeric nanoparticles (chitosan), liposomes, micelles, solid lipid nanoparticles, and gold nanoparticles are commonly used in the treatment of BC.[82, 83]

Due to their positive charge, chitosan nanoparticles (CHNPs) have great potential as a means of drug delivery that enables them to be transported across cell membranes and in sequential endocytosis.[84, 85] Their mucoadhesive attributes help in disentangling the epithelial tight junctions, which makes CHNPs suitable for oral administration.[86, 87] In addition, the presence of a free amino group facilitates CHNPs with targeting ligands for active targeting. Conjugated ligand CHNPs get away endo-lysosomal section and aggregate cytoplasm due to receptor-mediated endocytosis and release the drug for a longer period of time.[88-90] A. Yadav et al. (2020) produced a stable combination of raloxifene-encapsulated CHNPs and RGD-CHNPs by non-toxic ionic gelation. pH-dependent research revealed that nanoparticles have more stability, zeta potential, and cellular uptake at acidic pH (as in solid tumors) in comparison with physiological pH. RGD combination enhances in vitro cellular absorption of CHNPs in $\alpha v\beta 3$ integrin-expressing BC cells and induced more apoptosis in BC cells that was further augmented by lower pH. Furthermore, Rlx-RGD-CHNPs obviously inhibited the migration and angiogenesis in BC cells.[91] Z. Shakeran et al. (2021) developed a novel method to produce biodegradable mesoporous silica nanoparticles (MSNs) with tiny and identical particle sizes, achieve high methotrexate (MTX) loading through covalent amine and chitosan-functionalization, monitor cell uptake, and display the potential for reduced BC cell viability at low doses.[92] In their research, magnetic alginate/chitosan nanoparticles were created with curcumin loading to increase the bioavailability, uptake ability, and cytotoxicity of curcumin to human Caucasian BC cells (MDA-MB-231). They deposited alginate and chitosan on Fe3O4 magnetic nanoparticles based on their electrostatic attributes. The curcumin had sustained release by changing the number of layers of chitosan and alginate on the nanoparticles. The MTT assay and FACS assay indicated that the curcumin-loaded nanoparticles demonstrated significantly more cytotoxicity towards MDA-MB-231 cells than HDF cells.[93]

Uterine Cancer

Malignancy originating from endometrial glands is known as carcinoma, compared with the rare uterine sarcoma that originates in

mesenchymal tissues such as smooth muscle or connective tissue.[94] There are two types of endometrial cancer. Type I is more prevalent, making up more than 70% of cases. This kind of cancer is related to unopposed estrogen incitement and is known as endometriosis adenocarcinoma, which are some low-grade tumors. Type II tumors are more probable to be high grade, with a poor prognosis and a high risk of recurrence and metastasis. Only 10% of uterine cancers account for type II, which accounts for 40% of related deaths.[95, 96]

It was estimated that there were 14,480 new cases of uterine cervix cancer and 66,570 new cases of uterine corpus cancer in 2021. Uterine cancer (cervix and corpus) is the second most common gynecologic cancer among women in terms of incidence and mortality worldwide in 2021.[59,97, 98] Endometrial cancer is less common in premenopausal females, and most cases occur in women over 50 years of age.[99] Estrogen exposure during life is the basis of most risk factors. Early menarche, late menopause, obesity, and estrogen-generating tumors are related to an expanded risk of endometrial cancer. Prolonged estrogen exposure is a significant risk factor, causing incessant endometrial growth. As cells proliferate, the probability of mutations and endometrial cancer increases.[100-103] Nulliparity has a significantly worse prognosis.

During pregnancy, progesterone is the dominant hormone, and pregnancy-related agents may affect the biology of endometrial epithelial cells.[104] Research has shown that the risk of endometrial cancer increases in patients treated with tamoxifen, a selective estrogen receptor modulator used for BC treatment.[105, 106] Lynch syndrome, the most common hereditary colorectal carcinoma, and polycystic ovary syndrome increase the risk of uterine cancer to a great extent. Meanwhile, some more protective factors were noted in research, such as full-term pregnancy, breastfeeding, contraceptives, physical activities, alcohol, and smoking.[107-110] Tables 3 and 4 classify endometrial carcinomas and sarcomas.[111]

Diagnosis

There are some common clinical manifestations for early detection of endometrial cancer. The main early symptom for women diagnosed with endometrial cancer is irregular uterine (vaginal) bleeding. About 80% of women with endometrial cancer experience abnormal uterine bleeding.[112, 113] Nevertheless, menometrorrhagia and extended cycles of amenorrhea (≥6 months) after the age of 45 years should be evaluated. A significant percentage of endometrial cancers occur between ages 45 and 64 years. All postmenopausal bleeding should be monitored, especially if there are risk factors for endometrial hyperplasia or cancer.[114] Women who exhibit the above symptoms should undergo abdominal, speculum, and pelvic exams. Women older than 45 years should undergo endometrial sampling. Medical, family, and surgical history may be related to the disease.[115] Diagnosis of endometrial cancer under the age of 45 is rare. Unusual cervical cytology may be the first clue to uterine cancer, but it is not very accurate. Based on age, symptoms, and the presence of risk factors, endometrial evaluation is recommended.[111] The most appropriate diagnostic plan in cases with probable endometrial cancer is still controversial. There are some assessments available for investigating probable endometrial cancer, such as transvaginal ultrasound scanning (TVS), hysteroscopy, and endometrial biopsy.[116] TVS is an accurate, non-invasive, available, and cost-effective method that examines the thickness of the endometrial layer.[117] Ultrasound results indicate biopsy indication due to endothelial thickness.[118] The thickness of the endometrium should be 4 mm or less for a normal transvaginal ultrasound result. Following ultrasonography, saline infusion sono-hysterography can also be applied to assess the endometrium to obtain better images of structural alterations, especially when cases have polyps, submucosal fibroids, and endometrial hyperplasia. Extra information about endometrial thickness and irregularities and abnormalities may be provided by magnetic resonance imaging.[119] The uptake pattern in the tumor site by fluorodeoxyglucose PET can be detected in different kinds of tumors.[120] Nevertheless, the decisive diagnosis of endometrial carcinoma is by histological

biopsy.[115] Hysteroscopy is usually indicated for patients at high risk for endometrial cancer and cases in whom outpatient biopsy was insufficient or unable. Hysteroscopy can detect endometrial polyps and other ultrasound irregularities. A positive result in hysteroscopy increases the risk of cancer, while a negative result in hysteroscopy decreases the risk of cancer.[121] In uterine carcinoma diagnosis, specific factors have a little role. In part of the sarcomas, CA125 elevates, and in part of leiomyosarcoma, lactate dehydrogenase levels raise so that it is not very practical.[122, 123]

Treatment

Recently, minimally invasive surgery has been applied for surgical staging in cases with endometrial cancer.[124] Cases with metastatic conditions should undergo more aggressive surgery called radical hysterectomy, which involves the removal of the uterus, cervix, parametria, and upper vagina.[125] Based on the stage and existence of risk factors, the treatment of endometrial cancer after surgery continues. Cases are categorized into low, intermediate, and high-risk groups, and based on the risk rate, adjuvant therapy is done.[126, 127]

For adjuvant therapy, radiation therapy is a common method for preventing local recurrence. In high-risk cases, besides radiotherapy, chemotherapy (carboplatin and paclitaxel) accompanied by a considerable diminution in recurrence rate.[128] PORTEC-2 trial corroborates that vaginal brachytherapy is a standard adjuvant therapy for cases with high-intermediate recurrence risk.[129] In early-stage endometrial serous cancer, platinum-based chemotherapy in combination with bevacizumab, a VEGF inhibitor, as first-line adjuvant treatment is advised.[130, 131]

Nanotechnology is being used clinically to boost therapeutic indexes of chemoradiotherapy. Paclitaxel nanoparticle albumin-bound (nab), a new authorized particle-based chemotherapeutic, is recently being evaluated following its simultaneous prescription with radiotherapy in many chemoradiotherapy clinical trials (Phase III) in endometrial and cervical cancer.[132] Nano-based methods have demonstrated affirmative outcomes in these therapeutic areas.

A parallel therapeutic system to decrease the tumor conformity of a radiotherapy-resistant cell niche, boosted chemo-radiotherapeutics, boosted PET-CT contrast for designing/assessing response, and in vivo image contrast background to help image-guided therapies remain critical necessities.[133] Lately, a novel Nano self-assembled core-shell system micelle made by low molecular weight carboxymethyl chitosan and α-tocopherol succinate has been generated. The maximum tamoxifen load of the system can reach 8.08 ± 0.98%. The consistency of the system has been shown and the bioavailability has increased by 1.9-fold in comparison with that with free drug molecules.[134]

Some studies have applied folic acid combined with chitosan to generate nanocarriers to improve the drug loading performance and the bioavailability of chitosan. C. Misra et al. made tamoxifen practicable folic acid chitosan nanoparticles, where drug attaching is created by H-bonding, van der Waals bonding, and hydrophobic links. They have shown that as the measure of the nanocapsules elevated, a more firm drug-polymer bond was made, and TAM was more efficient.[135]

In a xenograft model of endometrial cancer, K. Ebeid et al. generated more lethality to paclitaxel (the first treatment for endometrial carcinoma) in cells with mutant p53 and increased the therapeutic effects applying polymeric nanoparticles. P53 observes checkpoints in the cell cycle as a supervisor of the genome, allowing cells to correct the damaged DNA or cause apoptosis. They prepared a composition of paclitaxel-loaded nanoparticles with the antiangiogenic molecular suppressor BIBF 1120 for amplifying the lethality specifically. Treatment resulted in significant inhibition of tumor progression and long survival.[136]

Chitosan-based polymeric nanoparticles application in pre-clinical and clinical research

Scientific studies have provided promising results from chitosan nanoparticles in anticancer drug delivery and cancer treatment. Nanodrug delivery systems based on CHPNPs have been developed for preclinical and clinical research.[137]

In this article, CHPNP's preclinical and clinical applications for recognition and cancer treatment will be discussed due to their less systemic toxicity and more cytotoxicity against cancer cells and tumors. Because of their specific characteristics, their applications include oral delivery, ocular drug delivery, nasal drug delivery, pulmonary drug delivery, mucosal drug delivery, gene delivery, vaccine delivery, vaginal drug delivery, and cancer treatment. Some of these studies are listed in table 5.

Conclusion and Future Perspective

Gynecological and BCs are the most important malignancies in women, negatively affecting the lifestyle of families and leading to many other ailments. Finding ways for early diagnosis and treatment can be a turning point in the fight against these cancers. Cancer cells could be detected and treated more efficiently by using nanotechnology. Novel drug delivery systems provide many promising methods to the challenges faced by kind of cancer treatment. In the treatment of gynecological cancers, nanocarriers help deal with challenges of low aqueous dissolution of chemotherapeutic medicines and more precise targeting either by active or inactive targeting, hence reducing adverse side effects. Chitosan-based polymeric nanoparticles are a favorable source for co-delivery of chemotherapeutic combinations for gynecological cancers. Drug resistance and cancer recurrence will be eliminated by using nanochemotherapeutics. According to the current review, nanotechnology provides many promising methods to the challenges faced by current cancer detection and treatment. In the treatment of cancers, nanocarriers help deal with challenges of low aqueous dissolution of chemotherapeutic medicines and more precise targeting either by active or inactive targeting, hence reducing adverse side effects. Chitosan-based polymeric nanoparticles have a new perspective for combined treatment strategies against cancers. They are a favorable source for co-delivery of chemotherapeutic combinations for selective treatment. Innovative therapeutic methods are made with the application of nanotechnology. Advances in early diagnosis and efficient noninvasive therapy in many types of cancers by applying nanotechnology have created clear horizons of increasing the chances of survival rate in these diseases. The use of chitosan nanoparticles in the diagnosis and treatment of gynecological cancers has yielded promising results, and further research in the clinical context is needed to precisely evaluate their effectiveness. It should be our constant effort to fight for the definitive cure and more survival advantages.

Conflict of Interest

None declared.

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Original Article
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Identification of Immunogenic Proteins in Early and Advanced Stages of Breast Cancer: An Immunoproteomics Study

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Abstract

Background: Early detection of breast cancer (BC) is extremely important as late diagnosis has been associated with a high rate of mortality. Immunogenic proteins and autoantibodies have been considered as favorable targets for early detection and targeted therapy in cancer. Accordingly, the present study aimed to identify the immunogenic antigens in both early and advanced stages of BC via a serologic proteome analysis (SERPA) approach.

Method: This is a case-control study wherein we separated the proteins from BC tissues in the early stages (n = 10) and advanced stages (n = 10) utilizing two-dimensional electrophoresis (2DE) and then transferred them onto a Polyvinylidene Difluoride (PVDF) membrane. To explore the tumor antigens reacting with antibodies, two-dimensional (2D) blots of tumor tissues in the early and advanced stages were separately probed with the sera from the same patients. Afterwards, we identified antibody-reactive proteins via liquid chromatography with tandem mass spectrometry (LC-MS/MS).

Results: Fibrinogen beta chain (FGB), protein deglycase DJ-1(PARK7), and peroxiredoxin-2 (PRDX2) were the highly reactive antigens identified in the early-stage patients. In addition, RuvB-like1 (RUVBL1) and triose phosphate isomerase (TPI) were recognized as the immune reactive proteins in the late-stage patients.

Conclusion: The results herein revealed that the immune-proteome pattern of BC patients changes along with tumor progression from primary to advanced stages. Moreover, immunogenic proteins seemed to stimulate the humoral immune system to produce autoantibodies in the initiation phase of BC; these autoantibodies could be employed as complementary factors for early detection of BC. The findings are however preliminary, and further studies with a larger sample size are required for verification and validation of previous findings.

Keywords: Breast neoplasms, Immunoreactive, Peptides, Autoantibodies, Serologic proteomic analysis, LC-MS/MS

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Introduction

Breast cancer (BC), as a prevalent malignancy, accounts for 18% of all female cancers, whose occurrence around the world is changing due to inevitable changes in life-style and advances in medical diagnosis tools.[1] This malignancy is one of the most frequent types of cancer in Iran, which has burdened the health-care system with a high annual cost. In this regard, an epidemiologic study revealed that BC among Iranian women is diagnosed at advanced stages with a lower mean age than the global average.[2] Due to the lack of early symptoms in the primary phase of breast cancer, it is usually diagnosed at higher stages; therefore, the treatment options are limited for BC patients and are associated with difficulties.[3] Moreover, early detection of breast cancer is extremely important since women whose breast cancer is detected at early stages have a higher survival rate in the first five years.[4] Immunogenic proteins and their cognate autoantibodies are the favorable targets for early detection and cancer immunotherapy.

During carcinogenesis, the proteins of normal cells undergo numerous variations, such as overexpression, altered glycosylation, and post-translational modifications (PTM), all of which could provoke the immune system into autoantibody generation.[5] The serum levels of cancer neo antigens are scarcely discoverable at the early stages of tumor progression, while a small amount of tumor-associated antigens (TAAs) can stimulate the immune system to produce antibodies.[6] The production of serum autoantibodies against TAAs has been observed in a variety of cancers, including B-cell acute lymphoblastic leukemia,[7] hepatocellular carcinoma,[8] colon cancer,[9] lung cancer,[10] prostate cancer,[11] breast cancer,[12] and primary open angle glaucoma.[13] Of note, the most important feature of circulating autoantibodies (AAbs), compared to classic tumor markers, is their detectability prior to manifestation of clinical symptoms.[14] In this regard, Chapman et al. observed that AAbs production against TAAs in breast carcinoma patients can be measured up to four years before tumor recognition by mammography.[15] Therefore, this remarkable data signifies the role of the immune system by recognizing the TAAs as "nonself" and provides a rapid humoral immune response in the early stages of BC. In the early stages of cancer, B cells, by class switching, can produce 5000–20,000 antibodies/min confronted with tumor-associated antigens.[16]

To date, several autoantibodies have been reported as BC biomarkers against known oncogenic proteins, such as p53, MUC1, HER2, and cyclin B1, with high degrees of diagnostic value.[17] In addition, a panel of autoantibodies against lesser-known immunogenic proteins has been reported in BC. These include ribosomal protein S6, eukaryotic elongation factor 2, eukaryotic stretching factor 2 kinase, heat shock protein 90 (HSP90), Ku protein, and

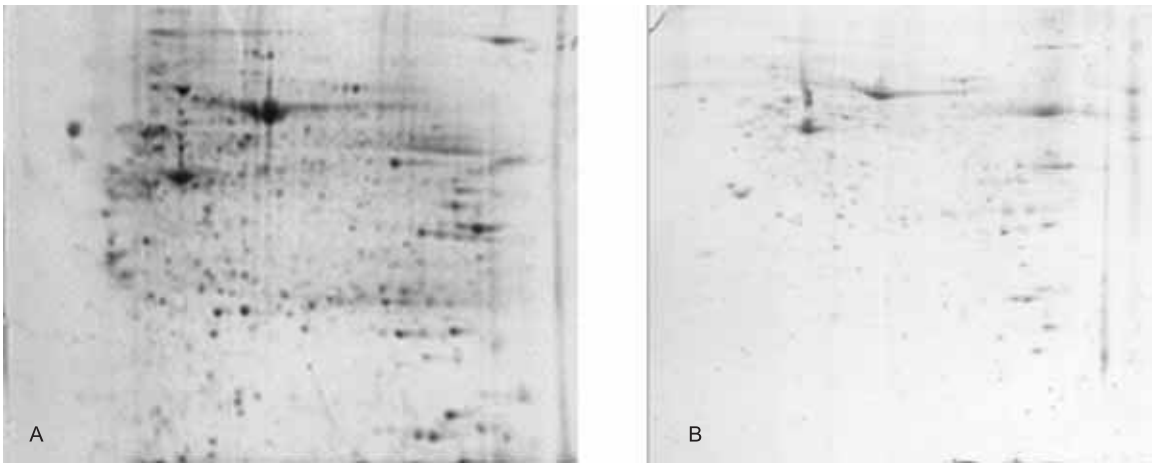

Figure 1. This figure shows the two dimensional electrophoresis (2-DE) proteome pattern of tumoral breast tissue (A) and normal breast tissue (B). The two dimensional (2D) gels were stained with Coomassie Brilliant Blue G-250.

topoisomerase I.[18] Immunogenic proteins on the other hand could be suitable targets for cancer diagnosis, prognosis, targeting, and vaccination.[19] Additionally, the autoantibodies against such proteins could be considered as potential serum biomarkers for early detection of BC.[18] Furthermore, serological proteome analysis (SERPA) is a high throughput method for identification of tumor antigens reacting with antibodies.[20] Since there is scarce information regarding immunogenic proteins in BC, the present study aimed to explore a panel of immunoreactive antigens for Iranian BC patients in both primary and advanced stages. To this end, we used 2D blots of breast tumor tissues lysates in different stages as sources of antigens and separately probed them with the sera from BC patients and healthy individuals. Immunoreactive proteins were then identified via liquid chromatography with tandem mass spectrometry (LC-MS/MS) analysis.

Materials and Methods

Tissues and sera specimens

The Ethics Committees of Babol University of Medical Sciences approved this case-control study (IR.MUBABOL.HRI.REC.1398.245). We also took written consent from all the participants for blood and tissue samples collection. The tissue samples of the BC patients were collected following an operation in the Surgery Department of Shiraz Central Hospital (MRI) and Faghihi Hospital (Shiraz, Iran) over six months. All the tumor tissues were the invasive ductal carcinoma (IDC) type, which we categorized in two groups (clinical stages I and II or early stages, n = 10; clinical stages III or advanced stages, n = 10). Concerning the subjects, this study included women aged between 32 and 60 years with tumor grades I, II, and III. At the time of diagnosis, the patients had not experienced distant metastasis. Moreover, normal breast tissues were taken from healthy women after cosmetic surgery (n = 5).

The first sera was collected from 20 patients with confirmed BC (female, median age of 32-60 years, early stages, n = 10; advanced stages, n = 10) prior to the surgery or any interventional treatments. A second set of sera was taken from healthy volunteers with no history of cancer or autoimmunity in themselves or their first-degree family members (n = 10, female, median age of 35-60 years). The final set of sera was collected from the patients with autoimmune diseases (confirmed systemic lupus erythematosus (SLE), n = 2). Both sera and tissue samples were then stored at -80˚C until use. Table 1 represents the clinicopathological characterization of the 20 newly diagnosed BC patients.

Lysates preparation

Primarily, we washed the tissue specimens with 1 ml cold phosphate-buffered saline (PBS) to remove the blood and necrotic tissues and then stored them in liquid nitrogen. The tissue samples (0.1 g) were then placed into a mortar and turned into powder using liquid nitrogen and a mortar handle. Afterwards, the samples were homogenized on ice in 1 ml of lysis buffer (7 M urea, 2 M thiourea, 4% 3-[(3-cholamidopropyl)

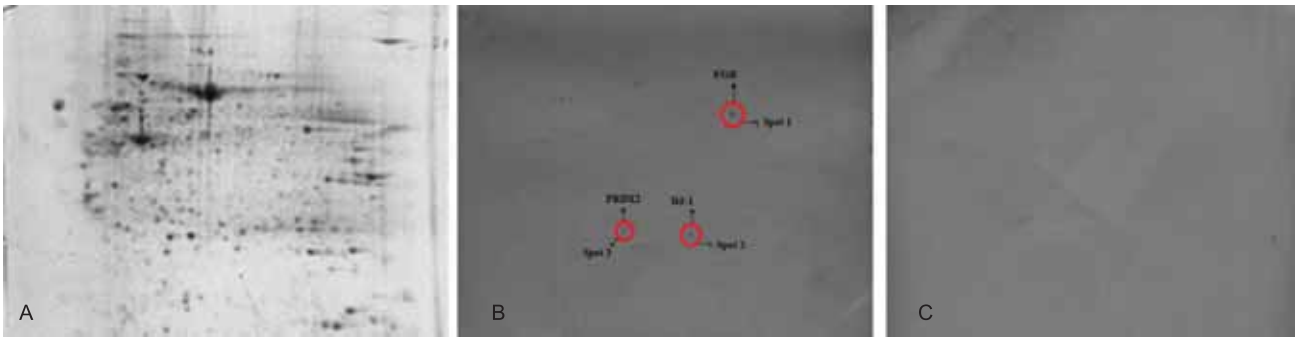

Figure 2. This figure represents the two dimensional (2D) gels and blots obtained from the tumor tissues lysates at an early stage; the gel stained with Coomassie Blue G-250 (A); the tumor tissue blots probed with the patients' sera at an early stage (B); the tumor tissue blots probed with the sera from the healthy controls (C). The blots were visualized by 3, 3'-diaminobenzidine (DAB) reagent. The spot number and identified proteins are related to table 2.

Table 1. Clinicopathological characterization of the patients with BC

Patient	Age	Tumor type	TNM stage	LVI	Tumor grade
P1	32	IDC	IA	negative	II
P2	42	IDC	IIA	positive	II
P3	45	IDC	IB	positive	II
P4	50	IDC	IIB	positive	I
P5	60	IDC	IA	negative	I
P6	48	IDC	IB	negative	II
P7	59	IDC	IIA	negative	III
P8	36	IDC	IIB	positive	III
P9	39	IDC	IIA	positive	II
P10	60	DC	IA	negative	I
P11	54	IDC	III B	positive	II
P12	48	IDC	III C	positive	II
P13	46	IDC	IIIA	positive	II
P14	39	IDC	III C	positive	II
P15	33	IDC	III B	positive	II
P16	47	IDC	III C	positive	II
P17	50	IDC	III A	positive	II
P18	60	IDC	III B	positive	II
P19	52	IDC	III C	positive	II
P20	59	IDC	IIIA	positive	II

LVI: lymphovascular invasion; TNM: Tumor, node, and metastasis; BC: Breast cancer; IDC: Invasive ductal carcinoma

dimethylammonio]-1-propanesulfonate (CHAPS), 2% immobilized pH gradient (IPG) buffer, 40mM dithiothreitol (DTT) in the presence of protease inhibitors. Homogenates were centrifuged at 14,000 × g for 15 min at 4°C. Subsequently, we collected the supernatant and determined the total protein concentration in each sample utilizing the Bradford method and aliquoted into 500 µl; they were kept at –80°C. All the components were purchased from GE Healthcare (Sweden).

Two-dimensional gel electrophoresis (2-DE)

2-DE is a combination of isoelectric focusing (IEF) and Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE), in which proteins are separated based on isoelectric point (PI) and molecular weight.[24]

2D western blot analysis

For 2D western blotting, we transferred the proteins from the 2D gels onto PVDF membranes with a semi-dry blotter (BioRad Laboratories, Hercules, CA, USA) under a voltage constant condition (22v) for 1 h. Thereafter, the PVDF membranes were blocked with 5% skimmed milk in PBS/Tween for 16 hours at room temperature. After blocking, the PVDF membrane was incubated with sera as a source of antibodies with

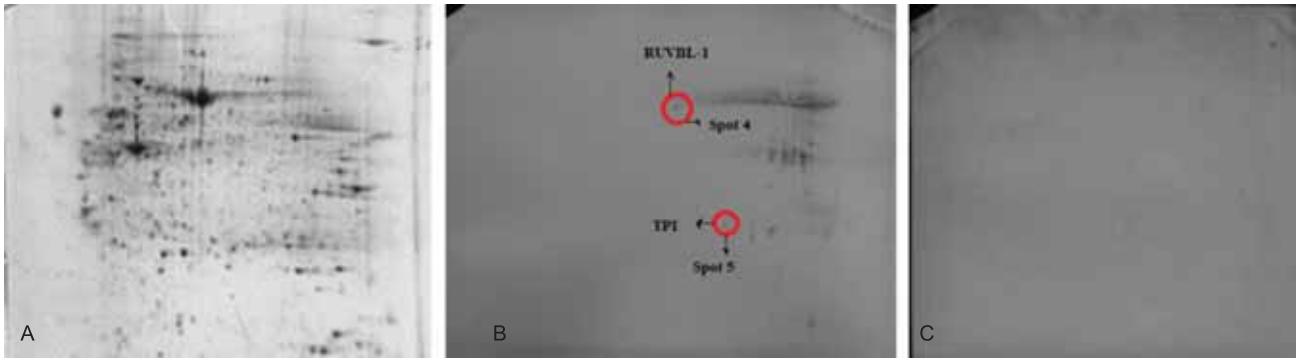

Figure 3. This figure represents the two dimensional (2D) gels and blots obtained from the tumor tissues lysates at a late stage; the gel stained with Coomassie Blue G-250 (A); the tumor tissue blots probed with the patients' sera at a late stage (B); the tumor tissue blots probed with the sera from the healthy controls (C). The blots were visualized by 3, 3'-diaminobenzidine (DAB) reagent. The spot number and identified proteins are related to table 2.

a dilution of 1:50 in blocking buffer with 2 h of incubation time. Subsequent to washing the PVDF membrane three times with PBS and 0.05% Tween-20 (PBST), we incubated it with goat anti-human IgG-HRP, as secondary antibody, with a dilution of 1:1000 in blocking buffer for 1 hour at RT on a shaker. The membrane was washed again three times with PBST and once with PBS.[21] The positive spots were then detected with 3,3'-Diaminobenzidine (DAB). We dissolved 0.05 gr DAB in 50 ml TBS followed by adding 72 µl H2O2. We then spilled it on the surface of the PVDF membrane. After 15 min, brown dots appeared at the location of the antigen-antibody reaction.

Spot selection

The PVDF membranes were scanned with a resolution of 300 dpi via a densitometer scanner (Bio-Rad Laboratories) and recorded in TIFF format. To map the spots with different immunoreactivities, we analyzed the blots using Prodigy software (version 1.0, Nonlinear Dynamics, Newcastle, UK). In addition, the matching process was confirmed by eye in at least three images.

Protein identification via LC - MS/MS

The protein spots reacting with cancer serum antibodies were manually cut from the 2D gels stained with Coomassie Brilliant Blue G-250. We rehydrated the gel pieces in a Trypsin/LysC solution. Digestion was carried out overnight at 37°C. Moreover, we purified the peptides via reversed phase extraction and analyzed them with LC-MS/MS. Statistical confidence limits of 95% were applied for the proteins. Cov (%) >53 were considered to indicate the statistically significant differences ($P < 0.05$). Furthermore, mass selection of the analyte with mass-to-charge ratio (m/z) was followed by fragmentation and analysis of the fragments. We then analyzed the produced tandem mass spectral data with the Protein Pilot software.

Protein-protein interactions and functional analysis

To survey the relationship between the identified proteins, protein–protein interactions (PPI) were analyzed via the STRING database (http://string-db.org). Moreover, the biological roles of the recognized proteins were interpreted by the GeneCards database (https://www.genecards.org) and the KEGG PATHWAY database (https://www.genome. jp/kegg/pathway.html).

Results

Bradford assay

We calculated the total protein concentration using Bradford assay; for the primary, advanced, and normal groups, 4.3 µg /µl, 6 µg /µl and 5.5 µg /µl were obtained, respectively.

2-DE proteome pattern

As shown in figure 1, despite overall similarities, the proteins' expression patterns indicated some differences between the tumoral and normal breast tissues. Figure 1 depicts the extracted proteins separated with 2-DE in the tumoral and normal breast tissues.

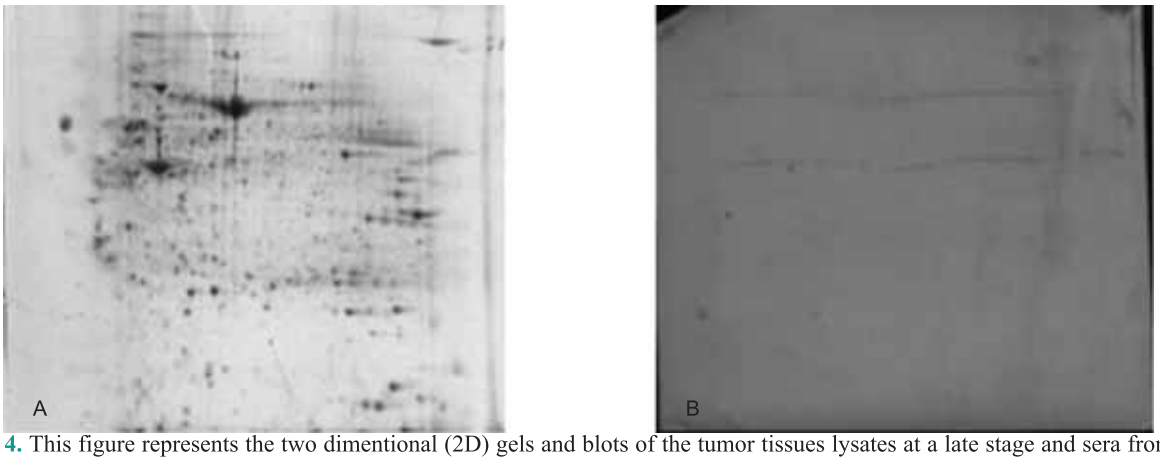

Figure 4. This figure represents the two dimentional (2D) gels and blots of the tumor tissues lysates at a late stage and sera from SLE patient; the gel stained with Coomassie Brilliant Blue G-250 (A); the tumor tissue blots probed with the sera from the SLE patient (B).
SLE: Systemic lupus erythematous

Table 2. Description of the identified proteins via mass spectrometry

Spot number	Protein name	Accession No.	COV (%)	Peptide(%)	pI	MW(kDa)
1	Fibrinogen beta chain	sp|P02675|	56	83	8.3	55.9
2	Protein deglycase	sp|Q99497|	87	38	6.7	19.8
3	Peroxiredoxin-2	sp|P32119|	75	54	5.7	21.8
4	RuvB-like 1	sp|Q9Y265|	87	30	6.02	50
5	Triosephosphate isomerase	sp|P60174|	75	69	6.45	26

MW: Molecular weight; pI: Isoelectric point; COV: Coverage; No.: Number

Differential immunogenic proteins

To identify the cancer-specific immunogenic proteins in different stages of BC, we probed 2D blots of tumor tissues lysates at early stages with the sera from the BC patients at early stages. We also investigated 2D blots of tumor tissues lysates at advanced stages with the sera from the BC patients at advanced stages. To identify the tumor proteins that only reacted with patients' sera, 2D blots of tumor tissue lysates were also probed with the normal match individuals' sera. Moreover, we performed the experiment in triplicate for each group.

Our results indicated that the immunoreactive proteins of our early-stage patients were different with those in advanced stages. In this regard, our results revealed the presence of at least three reactive spots at early stages and two reactive spots at advanced stages. On the other hand, the reactive spots derived from the tumor tissues of either early stages or late stages were reproducible and had no visibility or reactivity with the healthy individuals' sera. Figures 2 and 3 illustrate the results.

Additionally, 2D blots of tumor tissue lysates were probed with the sera from the SLE patients to further differentiate between the tumor-specific immunogens and tumor non-specific immunogens that react with ordinary autoantibodies. As shown in figure 4, we observed no visibility or reactivity with the sera of those with SLE. Finally, we investigated 2D blots of the normal tissue lysates with the healthy individuals' sera; the results showed no visibility or reactivity (Figure 5).

Protein identification via mass spectrometry

The spots representing reproducibility without visibility or reactivity with the sera from healthy individuals and SLE patients were sent for mass spectrometry analysis. Table 2 demonstrates the descriptions of the identified proteins. In general, we identified five highly reactive proteins in the BC patients; fibrinogen beta chain (FGB), protein deglycase DJ-1(PARK7), and Peroxiredoxin-2 (PRDX2) were the immune reactive antigens in the early-stage patients, whereas RuvB-like 1 and Triose phosphate isomerase (TPI) were recognized as the immune reactive biomarkers in those at late stages.

STRING analysis

The STRING database involves both well-

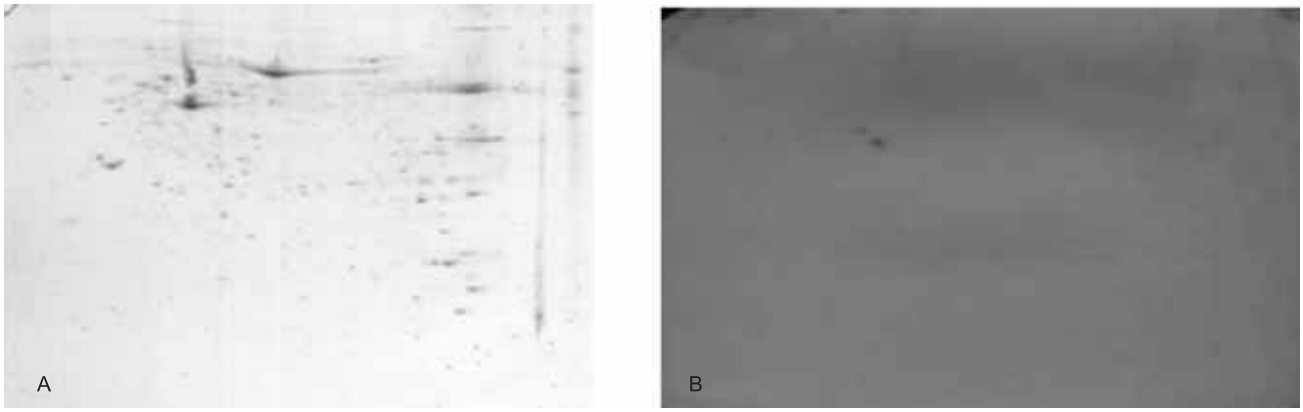

Figure 5. This figure represents the two dimensional (2D) gels and blots of the normal tissue lysates; the gel stained with Coomassie Brilliant Blue G-250 (A); the normal tissue blots probed with the sera from the healthy individuals (B).

known and predicted protein interactions in which genes or proteins are represented with nodes and edges marked with different colors. The functional linkages between the two proteins are ranked based on confidence score, with each score providing information about the functional similarity between the two proteins. The confidence score was previously reported as highest (0.9-1), high (0.7-0.8), medium (0.6-0.4), and low (0.1).[22] Figure 6 depicts the STRING analysis of the identified proteins. Based on STRING analysis, we herein calculated the confidence score of the identified proteins and their functional partners to be at 0.999, reflecting the maximum functional linkage between these proteins.

Molecular function and biological process of the identified proteins

We utilized the GeneCards and KEGG PATHWAY databases for investigating the molecular function and biological process of the identified proteins. The results are summarized in table 3.

Discussion

In the present study, we identified a panel of immunogenic proteins in BC patients in different stages of the disease. We also realized that the immunoproteomics pattern of the early-stage patients is different from that of the advanced-stage ones. These findings could be attributable to the changes in the expression pattern of proteins during tumor progression. In addition, the quality and severity of immune response varies from stage to stage in BC patients.[23]

Fibrinogen beta chain (FGB), DJ-1 (PARK7), and PRDX2 are the highly immunoreactive proteins identified with the LC- MS /MS approach in BC patients in early stages. Triosephosphate isomerase (TPI) and RUVBL1 are two of the other candidates identified for BC patients in advanced stages. As the immune system is an indispensable player during tumorigenesis and cancer development, autoantibodies, particularly cancer antigen-specific autoantibodies, may be used as early biomarkers for cancer detection and prevention. More importantly, the detection of these autoantibodies may be indicative of novel treatment strategies (development of monoclonal antibodies against the same cancer antigen to cure the disease). Functional analysis, as summarized in table 3, demonstrated that the identified proteins had functional similarities and participated in various biological processes, such

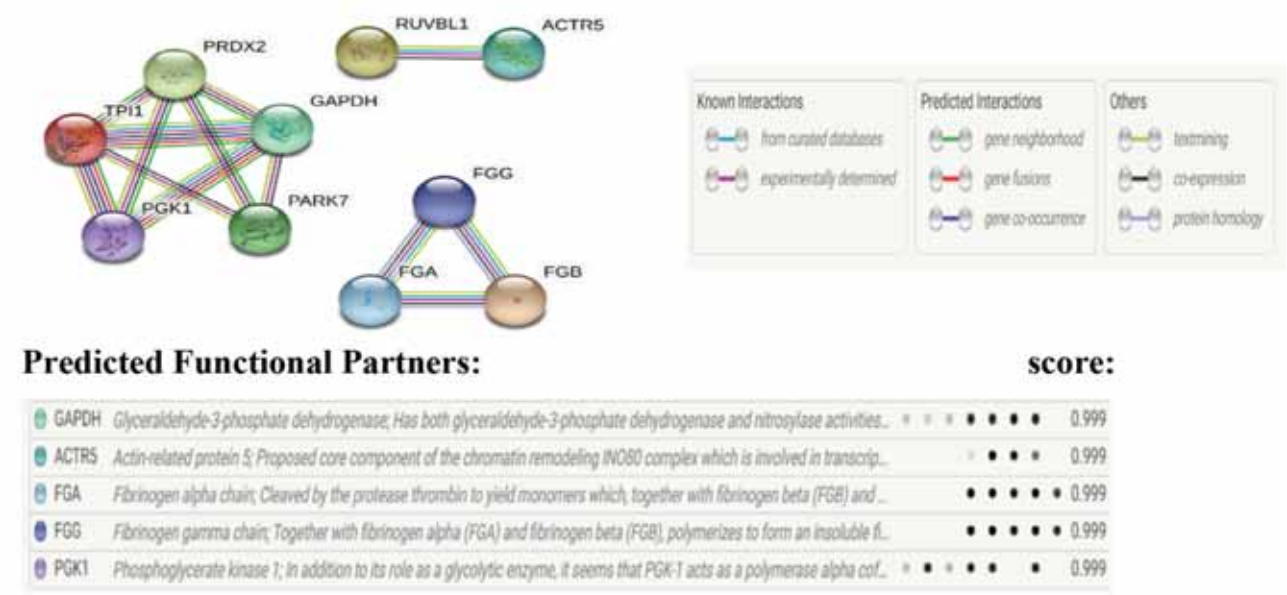

Figure 6. This figure shows the STRING analysis of the identified proteins. String analysis indicated a significant co-expression between Triosephosphate isomerase (TPI), Protein deglycase (DJ-1), and Peroxiredoxin-2 (PRDX2). Based on STRING analysis, both FGB and RUVBL1 proteins have an independent expression pattern compared to the other identified proteins.

Table 3. Functional analysis of the identified proteins

Protein	Molecular function	Pathways Proteins	interaction	Biological Process
PRDX2	Peroxidase activity Protein binding Antioxidant activity	Detoxification of ROS Glucose metabolism Neurodegenerative diseases		Activation of MAPK activity response to oxidative stress removal of superoxide radicals
TPI	Isomerase activity Protein binding Methylglyoxal synthase activity	Carbon metabolism Glucose metabolism		Gluconeogenesis Glycolytic process, Methylglyoxal biosynthetic
DJ-1 (PARK7)	Redox-sensitive chaperon Transcription coactivator Redox-sensitive protease DNA and RNA binding	PI3K / Akt signaling Parkinson disease Alpha-synuclein signaling		DNA repair Synaptic transmission
RUVBL1	ATPase activities DNA helicase activities Nucleotide binding Transcription coactivator	C-MYC pathway Wnt signaling pathway Chromatin regulation / Acetylation		DNA repair DNA recombination chromatin remodeling
FGB	Protein binding ECM structural constituent	Blood clotting cascade cell adhesion		Immune response Platelet degranulation

The protein interactions are according to GeneCards database. MW: Molecular weight; pI: Isoelectric point; COV: Coverage; PRDX2: Peroxiredoxin-2; RUVBL1: RuvB-like 1; DJ-1 (PARK7): Protein deglycase; TPI: Triosephosphate isomerase; PRDX2: Peroxiredoxin-2. ROS: Reactive oxygen species; MAPK: The mitogen-activated protein kinase

as glucose metabolism, DNA repair, redox hemostasis, cell adhesion, and blood clotting cascade. In this regard, STRING analysis exhibited a significant co-expression between TPI, DJ-1 (PARK7), and PRDX2.

An increased level of glycolysis is believed to be a remarkable property of cancer cells due to the extended energy requirement for cell proliferation. This feature is relatively assisted by upregulation of the glycolytic enzymes, such as Triosephosphate isomerase (TPI).[24] TPI catalyzes the reaction to convert dihydroxyacetone phosphate into glyceraldehyde 3-phosphate, and vice versa in glycolysis and gluconeogenesis. Our results revealed a high immunoreactivity between TPI and their cognate antibodies at advanced stages of the disease. Additionally, antibody production against TPI was previously reported in the early stages of BC.[25] The co-expression between TPI and early-stage immunogenic proteins (DJ-1 (PARK7) and PRDX2) in our study may clarify these proteins' tendency to show a coordination in expression and probably simultaneously participate in a biochemical pathway required for cancer progression. These findings may suggest that antibodies against TPI were continuously produced from the early stages of the disease, which can be detected even at higher stages.

Peroxiredoxins (PRDXs) are non-seleno peroxidases that catalyze the peroxide reduction of H2O2 and peroxynitrite. An elevated level of ROS in the early stages of BC leads to upregulation of PRDX2. Desmetz et al. reported that autoantibodies against PRDX2 could be used for early diagnosis of invasive BC.[12] In accordance with these results, we also observed that PRDX2 autoantibodies are generated in early stages of BC.

DJ-1(PARK7) protein, as an oxidative stress sensor, has exhibited a functional similarity to PRDX2. DJ-1 mRNA is overexpressed in different types of cancer, such as BC, and helps cancer cells to escape from PTEN enforced cell death.[26, 27] Furthermore, two previous studies illustrated a high level of DJ-1 protein in sera of BC patients.[28, 29] DJ-1 protein undergoes various post-translational modifications (PTM), among which O-linked N-acetylglucosamine is one of the effective PTMs, enhancing oncogenic activation of DJ-1 in primary stages of BC.[30] LC MS/MS analysis in this study revealed different PTMs in DJ-1 sequences, such as oxidation and carbamidomethylation. Overexpression of DJ-1 in BC tumor tissues and various post-translational modifications might lead to autoantibody

production against DJ-1(PARK7) in primary stages of BC.

Based on STRING analysis, both FGB and RUVBL1 proteins have an independent expression pattern compared with the other identified proteins. Fibrinogen is a plentiful protein synthesized in the liver, whose concentration ranges from 1.5-4 g/L in human blood plasma.[31] Extrahepatic tissues, such as cancer cells, can produce FGB, which leads to pathological conditions. The association between coagulation factors and cancer was previously proven.[32] It has been also reported that high plasma fibrinogen levels are associated with cancer development and progression.[33, 34] BC is characterized with fibrinogen deposition without further conversion to fibrin in the tumor stroma. This new finding about tumor cells' ability suggests that extrahepatic cell-derived FGB and plasma FGB may have specific functions in the process of various diseases, including BC. In 2019, Setareh Fayazfar et al. showed that FGB could be considered as a potential plasma biomarker for early diagnosis of colon cancer.[35] Consistently, we detected a high immunoreactivity between FGB and their cognate antibodies in the early stages of BC.

RUVBL1 acts as a DNA-dependent ATPase, belonging to the ATPases protein family.[36] It is mostly overexpressed in various cancers and plays a pivotal role in oncogenic processes.[37-39] An increase in the expression of RUVBL1 was reported in high metastatic BC cells, and silencing RUVBL1 consequently suppressed cancer cell development in both in vitro and in vivo models.[40] In this study, we realized that autoantibodies against RUVBL1 were produced in the late stages of BC probably due to the aberrant expression of RUVBL1. Research has exhibited that overexpression of RUVBL1 as an immunogenic protein might be directly related to tumor progression and development. Thus, RUVBL1 could be considered as a new candidate marker for BC targeted therapy, specifically in those with advanced stages.

Multiple BC-specific AAbs have been identified for early diagnosis of the disease. Although individual AAbs have shown poor performance for population-based screening, autoantibody panels have shown encouraging results. Modern screening digital mammography has a sensitivity of 86.9% for BC screening. None of the AAb panels reported so far for BC has qualified as standalone screening assays, but could be useful in combination with routine mammography screening. Moving these promising AAb candidates into clinical use necessitates a rigorous systematic approach. Proper study design, statistical models, extensive analytical and clinical validation, along with well-defined quantitative parameters are the necessary attributes to develop a useful AAb-based diagnostic screening tool for BC.

To date, researchers in the field of proteomics have mostly focused on comparing the proteomics pattern of BC patients with normal samples. Our study was nonetheless conducted using the SERPA method to identify immunogenic antigens in the sera sample of BC patients. Our work also detected these antigens and their autoantibodies, which can be used as a novel strategy for cancer therapy and early detection. Taken together, SERPA allows the simultaneous analysis of hundreds of proteins that are most intensely expressed in the tumor.

The technique of 2-DE; however, only detects the denatured proteins (linear epitopes) and the structural epitopes will be missed by the 2-DE. Moreover, this is a preliminary study, and the identified proteins as well as cognate antibodies should be verified with larger scale of BC patients by other methods, for instance, immunohistochemistry and ELISA, in order to explore their exact roles in early detection and clinical management of breast cancer.

Conclusion

In summary, our results revealed that immune-proteome pattern of BC patients changes along with BC progression from primary to advanced stages, which is related to the fact that severity of immune response varies from stage to stage in BC patients. Given the identification of three immunogenic proteins (FGB, PRDX2, and DJ-1) in the early stages of BC, these proteins could

stimulate the humoral immune system to produce antibodies in the primary phase of BC; thus, autoantibodies against them may be used as complementary factors for early detection of the disease. This study also demonstrated that RUVBL1 as a late-stage immunogenic protein may play a significant role in tumor development. Therefore, targeted therapy against this protein might be considered as a new promising approach to inhibiting tumor growth.

Acknowledgements

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Conflict of Interest

None declared.

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Original Article
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Improvement of NK Cell Cytotoxicity in Reconstituting NK Cells after Allogeneic Stem Cell Transplantation by Blocking NKG2A Checkpoint

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Abstract

Background: One cause of tumor relapse after allogeneic hematopoietic stem cell transplantation (allo-HSCT) is the alteration of the graft-versus-tumor effect of early reconstituting natural killer (NK) cells due to overexpression of the NKG2A inhibitory receptor. This study aims to determine the effect of Monalizumab, an anti-NKG2A receptor, on the effector functions of reconstituting NK cells after allo-HSCT.

Method: In this prospective cohort study, 18 patients with hematological malignancies were divided into three groups: dose 1 group (0.1 mg/kg, n = 5), dose 2 group (0.5 mg/kg, n = 8), and dose 3 group (1 mg/kg, n = 5), and followed up for six months. Blood samples were taken directly before the administration of Monalizumab and at different time points post-treatment. Reconstituting NK cells were phenotypically and functionally assessed by flow cytometry.

Results: Our results showed a more pronounced increase in the expression of activating NK receptors (NKG2D, NKp30, NKp46) on the reconstituting CD^{56dim} NK cells of patients receiving 1 mg/kg of Monalizumab compared with other participants. Additionally, we observed that patients treated with dose 3 of Monalizumab had the highest levels of degranulation compared with other patients and controls. Moreover, we noticed that CD^{56dim} NK cells of dose 2- and dose 3-related patients produced significant levels of perforin, interferon gamma (IFN-γ), and tumor necrosis factor alpha (TNF-α) in response to K562 stimulation post-Monalizumab treatment compared with controls and dose 1-treated patients.

Conclusion: We suggest that using Monalizumab improves the phenotype and cytotoxicity of reconstituting NK cells after allo-HSCT.

Keywords: Natural killer cells, Monalizumab, NKG2A, Cell cytotoxicity, Allogeneic hematopoietic stem cell transplantation

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Introduction

Allogeneic hematopoietic stem cell transplantation (allo-HSCT) is a promising alternative to

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chemotherapy for the treatment of hematological malignancies. However, it is associated with serious complications such as tumor relapse, infections, and graft-versus-host disease.[1-3] The immune system plays a critical role in tumor surveillance, and the performance of the patient's immune system after transplantation is a key determinant in allo-HSCT outcomes. One type of cell that plays a role in this process is natural killer (NK) cells, which are lymphocytes of the innate immune system that can recognize tumors and virus-infected cells without prior sensitization.[4,5] The function of NK cells is regulated by the expression of various inhibitory and activating receptors.[4,5] Additionally, NK cells exhibit antitumor activity through direct cytotoxic function and by regulating other immune cells through cytokine secretion. It has been observed that NK cells are the first lymphocytes to reconstitute following transplantation, and their rapid recovery is associated with lower relapse rates and improved survival.[6-8] However, early reconstituting NK cells are characterized by a high percentage of CD56bright immature NK cells, higher expression of the inhibitory receptor NKG2A, and variable expression of activating receptors, resulting in impaired cytotoxic function.[9-12]

NKG2A is an inhibitory receptor that belongs to the C-type lectin receptor family and recognizes non-classical HLA class I molecule, HLA-E.[13,14] It is expressed in association with CD94 on nearly 50% of the circulating NK cells. Furthermore, many types of tumor cells can evade NK cell immunity by upregulating HLA-E expression and increasing the expression of NKG2A on the surface of NK cells.[15,16] Therefore, blocking the NKG2A receptor with a monoclonal antibody could enhance the antitumor activity of reconstituted NK cells after hematopoietic stem cell transplantation (HSCT) and improve the cytotoxicity of NK cells in cancer patients.[16,17]

Monalizumab (previously known as IPH2201) is a humanized monoclonal antibody that targets the NKG2A inhibitory receptor.[18] Numerous studies have shown that the use of Monalizumab can restore and enhance the antitumor activity of NK cells in vitro and in humanized mouse

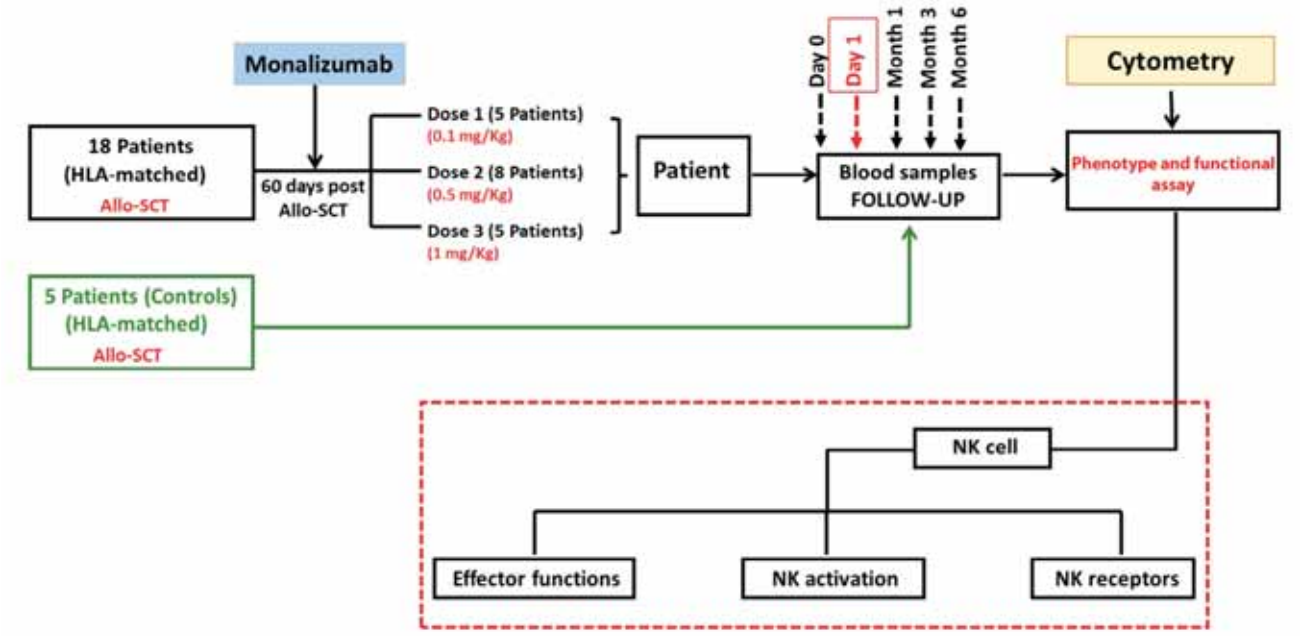

Figure 1. Time frame of the study: Patients suffering from hematological malignancies received allo-HSCT after achieving complete remission (CR) through chemotherapy. Monalizumab was infused into patients using an escalation dose regimen (3 doses). Peripheral blood samples were collected from both patients and controls at different time points. Subsequently, phenotype and functional assays were performed on the reconstituting NK cells.

HLA: Human leukocyte antigen; Allo-HSCT: Allogeneic hematopoietic stem cell transplantation; NK: Natural killer

models.[16,18,19] Moreover, several ongoing clinical trials are evaluating the safety and efficacy of Monalizumab in patients with different tumor types.

In the current study, three different doses of Monalizumab were administered to patients two months after allo-HSCT for hematological malignancies. Patients were monitored before and at various time points following Monalizumab administration to evaluate its effect on the functional activity of reconstituting NK cells.

Methods and Materials

Study design and patients

This is a prospective cohort study conducted at the Institute Paoli-Calmettes (IPC) in Marseille, France. The study adhered to the Helsinki Declaration criteria and received approval from the Ethics Committee of the Paoli – Calmettes Institutional Review Board under the ethical code of PIRAT-IPC 2019-018. A total of 18 patients with hematological malignancies were enrolled in the study. The sample size was calculated using

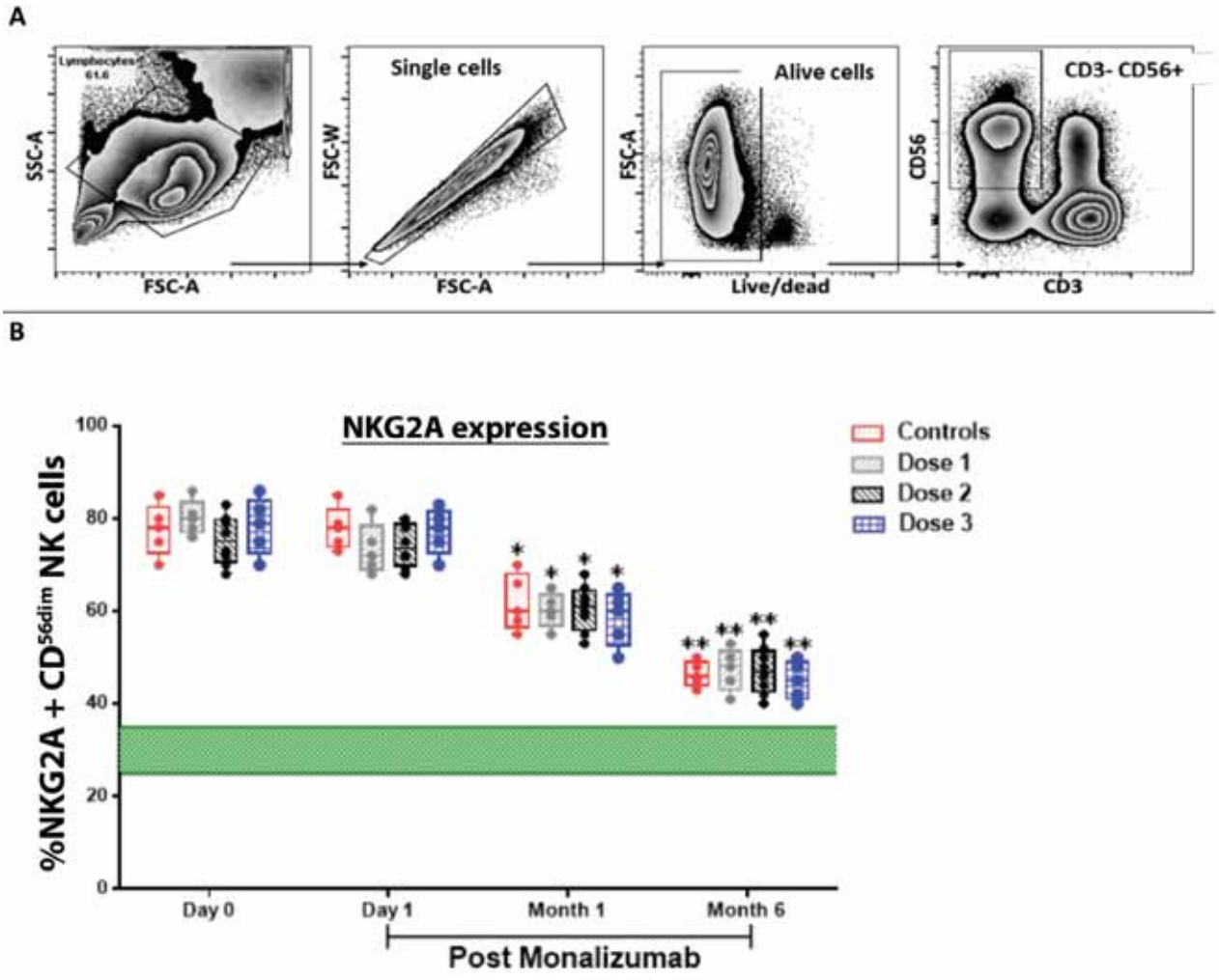

Figure 2. NKG2A expression profile on reconstituting CD^{56dim} NK cells after Monalizumab treatment: (A) A representative fluorescence-activated cell sorting (FACS) profile is shown, demonstrating the gating strategy for identifying NK cells. CD3-CD56+ NK cells were identified within PBMCs after gating for singlets and viable lymphocytes. (B) Box and whisker plots (minimum to maximum; horizontal lines represent median values) displaying NKG2A expression on reconstituting CD^{56dim} NK cells in the PB of patients treated with dose 1 (n = 5, gray boxes), dose 2 (n = 8, black boxes), and dose 3 (n = 5, blue boxes), as well as controls (red boxes), before and after different time points of Monalizumab treatment. Green lines represent the expression of NKG2A on NK cells in healthy donors. Comparisons between each pair of time points within the same group were performed using a non-parametric t-test (*$P \leq 0.05$, ** $P < 0.01$, *** $P < 0.001$).

NK: Natural killer; PBMCs: Peripheral blood mononuclear cells; PB: Peripheral blood

the formula for comparing means, with a confidence level of 95%, test power of 80%, and an effect size of approximately 0.8. All participants provided written informed consent in accordance with the Declaration of Helsinki.

The inclusion criteria were patients with hematological malignancies, including acute myeloid leukemia, acute lymphoblastic leukemia, multiple myeloma, chronic lymphoid leukemia, chronic myeloid leukemia, Hodgkin lymphoma or non-Hodgkin lymphoma, who had received allo-HSCT from HLA-matched (10/10) donors. The median age of the patients was 59 years (range: 39-68 years). Additionally, all patients received a reduced-intensity conditioning regimen consisting of fludarabine (30 mg/m^2/day), busulfan (3.2 mg/kg/day), and Thymoglobuline (5 mg/kg) for 2 days. They underwent HLA-identical allo-HSCT using granulocyte colony-stimulating factor-mobilized peripheral blood stem cells.

After 60 days post-transplantation, patients received intravenous Monalizumab according to a protocol approved by the ethical review boards at IPC. At day 60, Monalizumab was administered intravenously to patients in three dose groups: dose 1 group (0.1 mg/kg, 5 patients), dose 2 group (0.5 mg/kg, 8 patients), and dose 3 group (1 mg/kg, 5 patients). Peripheral blood samples were collected from patients at various intervals over a period of 6 months and cryopreserved for later

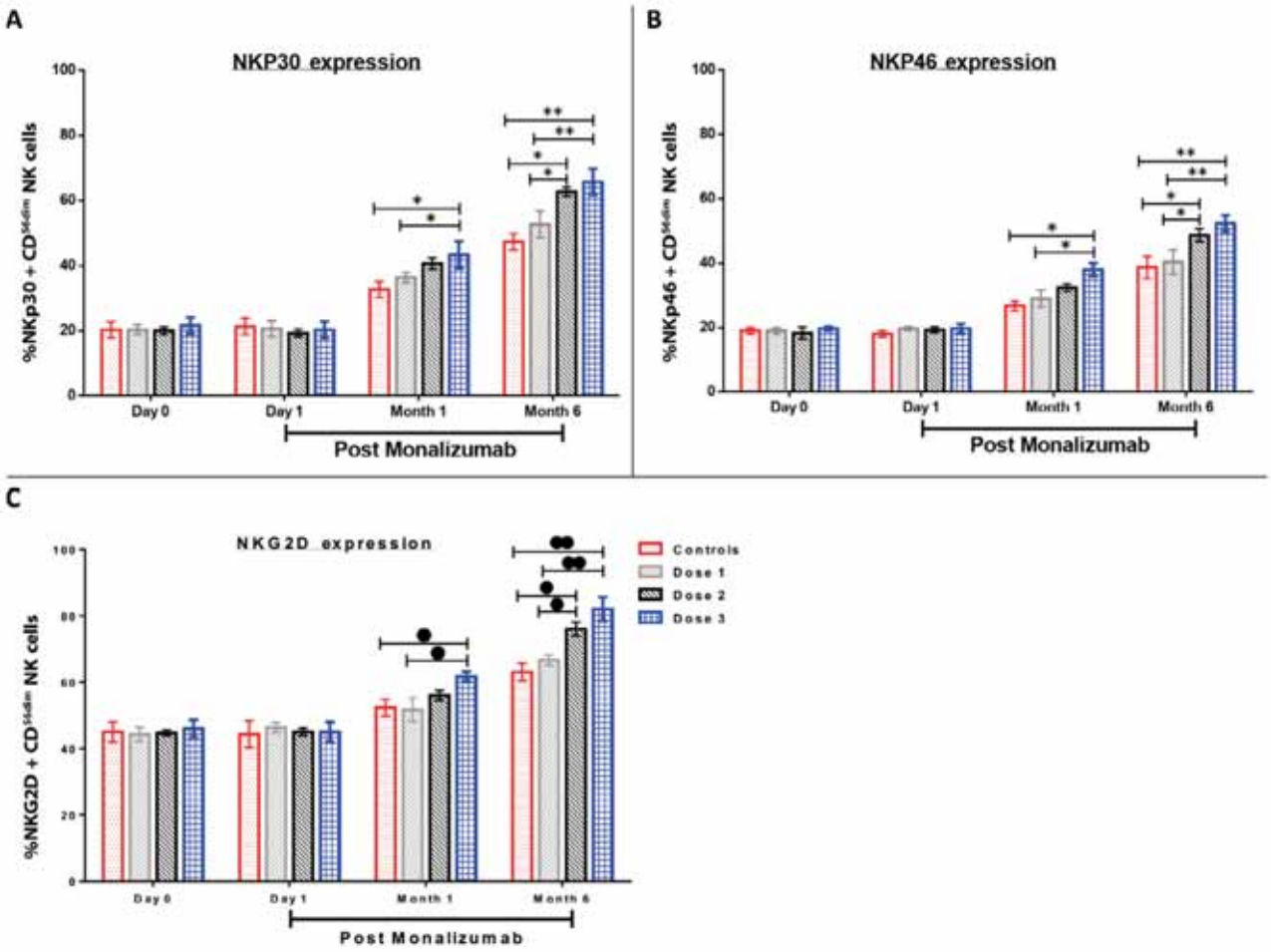

Figure 3. Activating NK cell receptors expression on reconstituting CD^{56dim} NK cells after Monalizumab treatment PBMCs were obtained from patients, stained with specific mAbs and analyzed by flow cytometry at different time points. The CD^{56dim} NK subset was identified within CD3-CD56+ NK cells. (A-C) Bar graphs display the mean ± SD of the frequencies of positive CD^{56dim} NK cells for NKp30, NKp46, and NKG2D receptors in PB of patients treated with dose 1 (n = 5, gray columns), dose 2 (n = 8, black columns), and dose 3 (n = 5, blue columns), as well as controls (red columns), before and after different time points of Monalizumab treatment. Comparisons between each pair of groups at the same time point were performed using a non-parametric Mann-Whitney U test (*$P \leq 0.05$, ** $P < 0.01$, *** $P < 0.001$).

NK: Natural killer; PBMCs: Peripheral blood mononuclear cells; mAbs: Monoclonal antibodies; PB: Peripheral blood

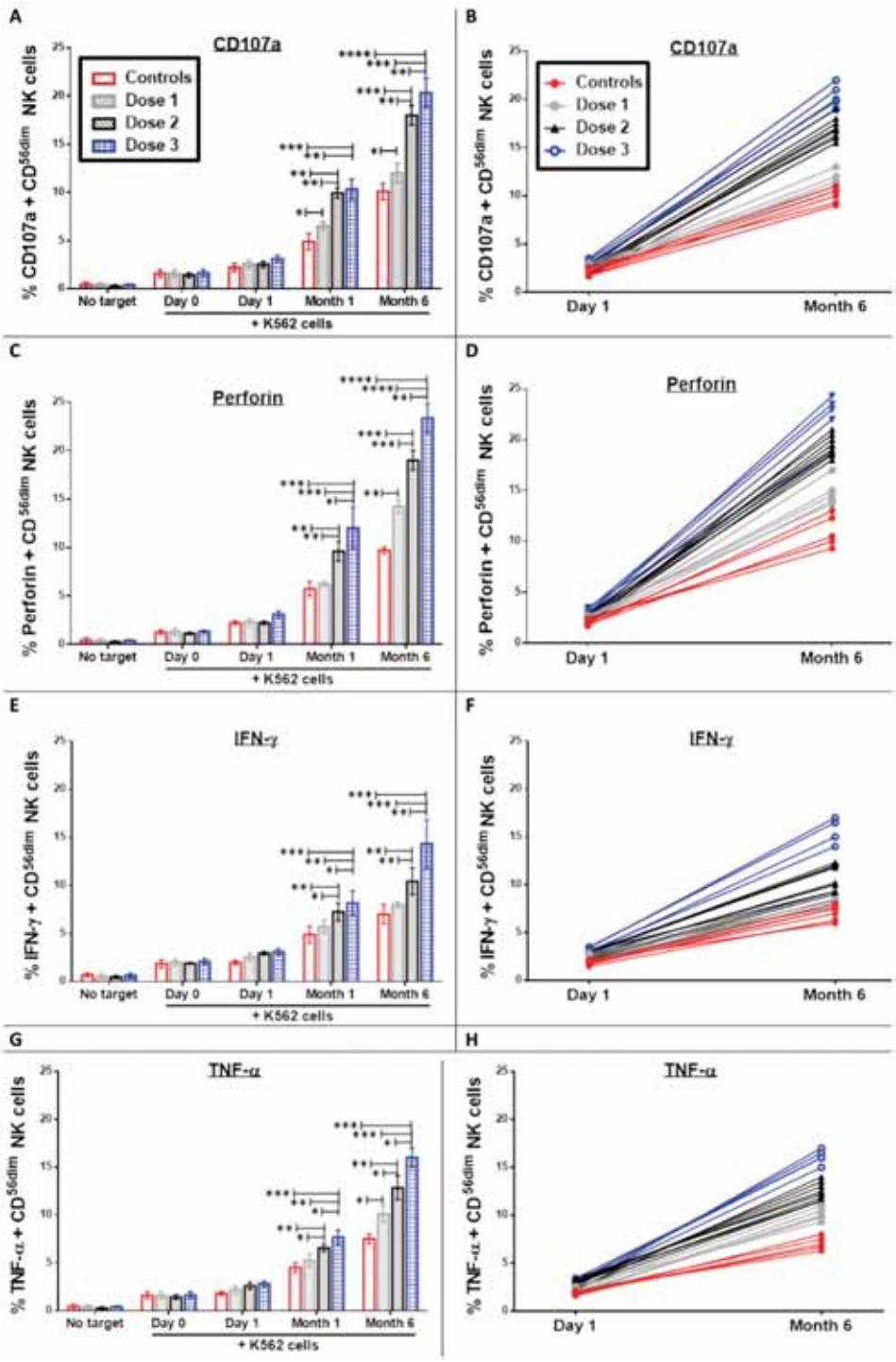

Figure 4. Effector function of reconstituting CD^{56dim} NK cells after Monalizumab treatment PBMCs from patients were collected at various time points and allowed to rest without cytokine activation. Resting cells were then cultured for 4 hours at 37°C, either alone or with K562 cells (E/T ratio, 10:1). After stimulation, the cells were stained for surface markers and intracellular molecules, and analyzed by flow cytometry. The CD^{56dim} NK subset was identified within the $CD^{3-}CD^{56+}$ NK cell population. (A, C, E, G) Bar graphs show the frequencies (mean ± SD) of CD^{107a+} CD^{56dim} NK cells (A), Perforin+ CD^{56dim} cells (C), IFN-γ+ CD^{56dim} cells (E), and TNF-α+ CD^{56dim} NK cells (G) in the dose 1 group (n=5, gray columns), dose 2 group (n=8, black columns), dose 3 group (n=5, blue columns), as well as controls (red columns), before and after different time points of Monalizumab treatment. (B, D, F, H) Displaying the chang es in frequencies of CD^{107a+} CD^{56dim} NK cells (B), Perforin+ CD^{56dim} cells (D), IFN-γ+ CD^{56dim} cells (F), and TNF-α+ CD^{56dim} NK cells (H) from day 1 to month 6 in dose 1 recipients (gray lines), dose 2 recipients (black lines), dose 3 recipients (blue lines), and controls (red lines). Comparisons between day 1 and month 6 were performed using the Wilcoxon non-parametric t-test. Comparisons between each two groups at the same time point were performed using the non-parametric Mann-Whitney U test(*$P \leq 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$).

NK: Natural killer; IFN-γ: Interferon gamma; TNF-α: Tumor necrosis factor alpha

use. The samples were obtained directly before Monalizumab treatment (Day 0, baseline) and at month 1, month 3, and month 6 after Monalizumab treatment (Figure 1). Genoidentical controls were used, and peripheral blood samples corresponding to the patients' time frame samples were utilized. Patients with a history of grade ≥ II acute graft-versus-host disease (GVHD), a history of another malignancy, abnormal cardiac status, previous allo-HSCT or solid organ transplantation, or ongoing use of systemic corticosteroids were excluded from the study. Additionally, five HLA-genoidentical transplanted patients were used as controls.

Flow cytometry

After thawing, the cells were immunostained with fluorochrome-labeled antibodies from phenotypic panels listed in table 1. Acquisition was performed using a FACS LSR II (BD Biosciences), and analysis was done using FlowJo v10 software (LLC, Ashland, Oregon). The gating strategy was based on the elimination of doublets using FSC-A/FSC-H parameters, followed by the removal of dead cells using a cell viability marker. The NK cell population was defined as $CD^{3-}CD^{56+}$ cells within the lymphocyte gate. The phenotypic data are represented as the percentage of cells positive for a given marker.

NK cell functions

PBMCs at all time points were thawed and rested overnight at 37°C in RPMI 1640 medium supplemented with 10% FCS (complete medium) without interleukins (IL-2 or IL-15). Cytokine production and degranulation capacity were determined by stimulating NK cells with the erythroleukemia cell line K562. Briefly, $1x10^6$ PBMCs were incubated with K562 cells (ratio 10:1) at 37°C and 5% CO_2 for 4 hours in the presence of GolgiPlug® (BD Biosciences, #555029) and FITC-conjugated anti-human CD107a (BD Biosciences, clone H4A3). After 4 hours of incubation, the cells were collected, washed, and immunostained for surface markers with fluorochrome-labeled antibodies listed in table 1. For intracellular staining, cells were fixed and permeabilized using the Cytofix/Cytoperm® kit (BD Biosciences) according to the manufacturer's instructions, and then immunostained with fluorochrome-conjugated anti-cytokine antibodies listed in table 1. Finally, the cells were prepared for flow cytometry analysis using a FACS LSR II (BD Biosciences) and FlowJo v10 software (LLC, Ashland, Oregon).

Statistical analysis

Graphics were generated using GraphPad Prism software (San Diego, CA, USA). Statistical analyses were performed using GraphPad Prism software. Comparisons between two different groups were conducted using the Mann-Whitney test, while comparisons between the same individual in each group were carried out using the Wilcoxon matched pairs T test. A significance level of $P \leq 0.05$ was considered statistically significant.

Results

Normal pattern of NKG2A expression on the reconstituting CD^{56dim} NK cells following Monalizumab treatment

To determine the expression of NKG2A on reconstituting CD^{56dim} NK cells following Monalizumab treatment, peripheral blood mononuclear cells (PBMCs) of patients before and after relevant time points of Monalizumab usage were stained with specific monoclonal antibodies (mAbs) and analyzed by cytometry. NKG2A expression was also assessed among control patients at the same time frames as the treated patients.

Firstly, NK cells were identified within PBMCs as $CD^{3-}CD^{56+}$ cells among viable lymphocytes (Figure 2A). Since early reconstituted NK cells display an immature phenotype characterized by high expression of NKG2A, our results showed an increase in the frequencies of $NKG2A+CD^{56dim}$ NK cells at day 0 and day 1 in all patients and controls, compared with healthy individuals. However, when compared with day 0, the frequencies of $NKG2A+CD^{56dim}$ NK cells began to significantly decline throughout the study period. At month 6, the frequencies of $NKG2A+CD^{56dim}$ NK cells were significantly lower compared with day 0 (controls: 45% at month 6 versus 78% at day 0, $P < 0.01$; dose 1:

Table 1. List of antibodies used for flow cytometry in the study

Activating NK cell receptors panel		
mAb	**Clone**	**Company**
Live/dead discriminator	Fixable aqua dead cell stain kit	Life technologies
CD335 (NKp46)-ef450	9E2	eBioscience
CD337 (NKp30)-PE	AF29-4D12	Miltenyi Biotec
CD314 (NKG2D)- PerCP-efluor710	1D11	eBioscience
CD56-PE-Vio770	AF12-7H3	Miltenyi Biotec
CD3-BV605	SK7	BD Biosciences
CD19-PE-CF594	HIB19	BD Biosciences
CD14-AF700	M5E2	BD Biosciences
CD16-APC-Vio770	VEP13	Miltenyi Biotec
NK Cell Functional Panel		
mAb	**Clone**	**Company**
Live/dead Discriminator	Fixable aqua dead cell stain kit	Life technologies
CD107a-FITC	H4A3	BD Biosciences
CD56-PE-Vio770	AF12-7H3	Miltenyi Biotec
CD3-PE-CF594	UCHT1	BD Biosciences
CD16-APC-Vio770	VEP13	Miltenyi Biotec
TNF-α-ef450	MAb11	eBioscience
IFN-γ-AF700	B27	BD Biosciences
Perforin-PE	B-D48	Biolegend

CD: Cluster of differentiation; ef450: eFluor 450; PE: Phycoerythrin; PerCP-eFluor710: Peridinin chlorophyll-eFluor 710; PE-Vio770: Phycoerythrin violet 770; BV605: Brilliant violet 605; PE-CF594: Phycoerythrin Cyanine-based Fluorescent 594; AF700: AlexaFluor 700; APC-Vio770: Allophycocyanin-violet 770; FITC: Fluorescein-5-isothiocyanate; TNF-α: Tumor necrosis factor alpha; IFN-γ: Interferon gamma

48% at month 6 versus 80% at day 0, $P < 0.01$; dose 2: 46% at month 6 versus 77% at day 0, $P < 0.01$; dose 3: 45% at month 6 versus 79% at day 0, $P < 0.01$) (Figure 2B).

Furthermore, we did not observe any variations in NKG2A expression between Monalizumab recipients and the controls at a given time point. The study population exhibited a similar NKG2A expression profile at the same time point ($P > 0.05$). These findings suggest that the use of Monalizumab did not alter the normal pattern of NKG2A expression on reconstituting CD^{56dim} NK cells.

Top of form

Monalizumab increases the expression of activating NK cell receptors on reconstituting CD^{56dim} NK cells

Next, we investigated whether the use of Monalizumab affected the expression of NK cell activating receptors by measuring the frequencies of NKp30, NKp46, and NKG2D activating receptors on the reconstituting CD^{56dim} NK cells in the study population at different time points. Our results showed that there were no changes in the expression of NK cell activating receptors among study participants before using Monalizumab and at day 1 of Monalizumab use ($P > 0.05$) (Figure 3A, B, and C). Additionally, the expression of NKp30, NKp46, and NKG2D remained consistent among the controls and patients in the dose 1 group throughout the study period ($P > 0.05$).

Interestingly, the frequency of $NKp30^{+}$ CD^{56dim}, NKp46+ CD^{56dim}, and NKG2D+ CD^{56dim} NK cells significantly increased after one month of using Monalizumab in patients who received dose 2 and dose 3 of Monalizumab compared with baseline, as well as control subjects ($P < 0.05$). Moreover, the increase was more pronounced after 6 months of using Monalizumab among patients treated with dose 3 of Monalizumab (frequency of NKp30+ CD^{56dim} at month 6: 65% in dose 3 versus 47% in controls, $P < 0.01$; 51% in dose 1, $P < 0.01$; 62% in dose 2, $P > 0.05$; frequency of NKp46+ CD^{56dim} at month 6: 52% in dose 3 versus 38% in controls, $P < 0.01$; 40% in dose 1, $P < 0.01$; 48% in dose 2, $P > 0.05$; frequency of NKG2D+ CD^{56dim} at month 6: 82% in dose 3 versus 63% in controls, $P < 0.01$; 67% in dose 1, $P < 0.01$; 78% in dose 2, $P > 0.05$).

However, we did not find a significant change in the expression of activating NK cell receptors between patients in the dose 2 and dose 3 groups

at the corresponding time points ($P > 0.05$). Taken together, these findings suggest that blocking NKG2A receptors using Monalizumab could upregulate the expression of NK cell activating receptors on the reconstituting CD^{56dim} NK cells.

Blocking NKG2A enhances the cytolytic function of reconstituting CD^{56dim} NK cells

In this study, we aimed to assess the impact of NKG2A blockade using Monalizumab on the effector function of reconstituting CD^{56dim} NK cells. We evaluated this by monitoring the cell surface expression of CD^{107a}, perforin secretion, and cytokine production (interferon gamma, IFN-γ, and tumor necrosis factor alpha, TNF-α) of reconstituting CD^{56dim} NK cells co-cultured with K562 target cells expressing HLA-E. Our results revealed that patients treated with doses 2 and 3 of Monalizumab exhibited higher levels of degranulation in reconstituting CD^{56dim} NK cells compared with patients treated with dose 1 and controls.

Moreover, the increase in degranulation levels was particularly significant in patients treated with dose 3, especially after 6 months of Monalizumab use. At month 6, the percentage of CD^{107a}+ CD^{56dim} NK cells was 23.5% in dose 3-treated patients, whereas it was 19.2% in dose 2-treated patients ($P < 0.05$), 14.4% in dose 1-treated patients ($P < 0.0001$), and 10.1% in controls ($P < 0.0001$) (Figure 4 A, B). Similarly, Monalizumab treatment resulted in increased perforin secretion by reconstituting CD^{56dim} NK cells, primarily in patients treated with dose 3. At month 6, the percentage of perforin+ CD^{56dim} NK cells was 20.3% in dose 3-treated patients, while it was 18% in dose 2-treated patients ($P < 0.01$), 12% in dose 1-treated patients ($P < 0.0001$), and 9.6% in controls ($P < 0.0001$) (Figure 4 C, D).

Regarding cytokine production, CD^{56dim} NK cells from patients treated with doses 2 and 3 exhibited significant levels of IFN-γ and TNF-α cytokines in response to K562 stimulation post-Monalizumab treatment, compared with controls and dose 1-treated patients. This change was particularly pronounced after 6 months of Monalizumab use (Figure 4 E-H). Interestingly, we observed that patients receiving dose 3 of Monalizumab had higher frequencies of IFN-γ^+ CD^{56dim} and TNF-α^+ CD^{56dim} NK cells compared with patients receiving dose 2, especially in the 6^{th} month of treatment (% IFN-γ^+ CD^{56dim}cells at month 6 in dose 3-treated patients was 14.3% compared with 10.2% in dose 2-treated patients, $P < 0.01$), and (% TNF-α^+ CD^{56dim} NK cells at month 6 in dose 3-treated patients was 16.2% compared with 12.7% in dose 2-treated patients, ($P < 0.05$)).

Taken together, these findings suggest that blocking NKG2A receptors using Monalizumab after allo-HSCT may enhance the cytotoxic activity of reconstituting CD^{56dim} NK cells.

Discussion

In the current study, we discovered that blocking the inhibitory function of NKG2A using Monalizumab after allo-HSCT led to improved NK cell restoration and increased expression of NK cell activating receptors on the reconstituting CD^{56dim} NK cells. Consequently, the effector function of these cells was enhanced. This improvement in both the morphological and functional properties of the reconstituting NK cells was particularly evident among patients treated with dose 3 of Monalizumab. HSCT patients undergo an aplastic period which can lead to infections and tumor relapse in immune-compromised recipients. Therefore, it is crucial for donor-derived HSCs to engraft rapidly and for immune cells to recover quickly in order to achieve positive clinical outcomes from HSCT.[20]

NK cells are the first donor-derived lymphocytes to regenerate following HSCT, serving as the initial protective line in immune-compromised recipients.[21,22] They are cytotoxic lymphocytes involved in innate immune responses, recognizing and eliminating virus-infected and tumor cells without prior stimulation. Clinical studies have demonstrated that NK cell-based immunotherapy represents a promising strategy for potent antitumor effects against hematological malignancies.[23-27] However, it has been shown that early reconstituting NK cells after HSCT have an immature phenotype

characterized by high expression of NKG2A, which is associated with diminished cytotoxic activity. This is because the balance between activating and inhibiting receptors precisely regulates NK cell behavior.[12,28-30] Furthermore, certain types of cancer highly express HLA-E, a specific ligand of NKG2A inhibitory receptors. As a result, these phenotypic abnormalities can hinder the effector functions of reconstituting NK cells, increasing the risk of tumor relapse and mortality.

In this study, we aimed to improve the functional activity of reconstituting NK cells after allo-HSCT in patients with hematological malignancies by blocking the NKG2A checkpoint using Monalizumab. Immune checkpoint inhibitors have generally had a positive impact on the management of many types of cancer; however, the efficacy of many of these inhibitors still needs to be further improved. Moreover, there have been very limited studies evaluating the effect of Monalizumab on the immune system and cancer cells. Unfortunately, we did not find any studies that assessed the effect of Monalizumab on the biology of NK cells. Nevertheless, our findings demonstrated that the maturation pattern and expression of activating receptors were improved in reconstituting NK cells. After six months of Monalizumab treatment, patients receiving dose 3 of Monalizumab experienced a significant decrease in the expression of NKG2A receptors and a remarkable increase in the frequency of $NKp30^{+}$, $NKp46^{+}$, and $NKG2D^{+}$ CD^{56dim} NK cells compared with baseline and other study participants at the relevant time periods.

The exact mechanism by which Monalizumab upregulates the expression of NK cell activating receptors is not well known. However, we believe that blocking NKG2A receptors diminishes the inhibitory signals that may be involved in inhibiting the gene transcription of the activating NK cell receptors. Additionally, it reduces the secretion of certain cytokines that may downregulate the expression of activating NK cell receptors.

Previous studies have reported that tumor cells can evade NK cell immunosurveillance by altering the phenotypic properties of NK cells. This is achieved through upregulation of NKG2A receptors and ligands, downregulation of NK cell activating receptors and their ligands, as well as the secretion of cytokines by tumor cells.[31-34]

Therefore, we hypothesize that by inhibiting the primary inhibitory NK cell receptors (NKG2A) and enhancing the expression of NK cell activating receptors, Monalizumab can potentially prevent tumor progression and relapse.

Based on the aforementioned progression in morphological features of early developing NK cells, we evaluated whether these changes would affect the functional activity of NK cells. Additionally, it has been reported that early reconstituting NK cells after HSCT have diminished cytotoxic function.[12,30,35] Our findings revealed that peripheral CD^{56dim} NK cells from patients receiving higher doses of Monalizumab displayed a higher degree of degranulation and greater proinflammatory cytokine secretion in response to K562 cell stimulation than CD^{56dim} NK cells from patients receiving dose 1 of Monalizumab or from controls. Notably, the increase was more pronounced for patients treated with dose 3 of Monalizumab. In agreement with our results, Andre et al. found that blockade of NKG2A in vitro with Monalizumab promoted the expression of CD107a and increased cytokine secretion by NK cells in response to target cell stimulation.[19] Additionally, they showed that Monalizumab improved NK cell-mediated antibody-dependent cell-mediated cytotoxicity (ADCC) when combined with cetuximab, an anti-epidermal growth factor receptor.[19] On the other hand, Tinker and his colleagues found that Monalizumab monotherapy had very little clinical activity in patients with gynecologic cancers.[36] However, using Monalizumab in combination with anti-PD-L1 in vivo enhanced the antitumor activity of $CD8^{+}$ T cells in a murine model of cancer, suggesting that using Monalizumab in combination with checkpoint inhibitors could provide synergistic anti-tumor effectiveness.[19,37] Given that prior studies have shown mature CD^{56dim} NK cells to be cytotoxic cells and the

major source of proinflammatory cytokines and chemokines after target cell stimulation, we believe that the functional activity improvement of reconstituting CD^{56dim} NK cells may be attributed to the blockade of NKG2A inhibitory function, as well as the significant recovery of NK cell maturity following Monalizumab treatment. Moreover, the upregulation of activating receptor expression on circulating CD^{56dim} NK cells may also contribute to the improved functional activity of reconstituting CD^{56dim} NK cells after treatment with Monalizumab.[38,39]

Our study has some limitations, such as a small sample population, a short follow-up period, and the fact that we did not consider NK cell infiltration into the tumor microenvironment or interactions with other immune cells that might alter the activity of Monalizumab. Additionally, it is essential to evaluate the expression of NK cell activation markers, as well as exhaustion markers, on the reconstituting NK cells before and after using Monalizumab.

Conclusion

In conclusion, we report here the benefits of using Monalizumab as a therapeutic antibody that enhances the antitumor activities of reconstituting CD^{56dim} NK cells by blocking the inhibitory function of NKG2A, as well as increasing the expression of NK cell activating receptors. Therefore, anti-NKG2A mAb is a promising checkpoint inhibitor that promotes antitumor immunity by enhancing the cytotoxic activity of NK cells. These results suggest that additional clinical trials using Monalizumab as monotherapy or in combination with other immune agents are necessary.

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Conflict of Interest

None declared.

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Original Article
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Comparison of Carcinoembryonic Antigen Level and E-Cadherin Expression between Metastatic and Non-Metastatic Colorectal Carcinoma in RSUP, Dr. Sardjito Yogyakarta-Indonesia

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Abstract

Background: WHO has reported 34,189 (8.6%) colorectal carcinoma cases out of 396,914 total cancer cases in Indonesia. Accumulated gene mutation and the environment can affect cell regulation, growth, and differentiation, impacting the methylation of tumor suppressor genes. Carcinoembryonic antigen (CEA) is a biomarker used to detect the presence of colorectal carcinoma. Moreover, the E-cadherin gene has an essential role in tissue homeostasis, the adhesion between cells at embryogenesis, tissue morphogenesis, differentiation, and carcinogenesis stages. During instability and dysfunction in its regulation, the E-cadherin gene induces tumor progression. This study aimed to compare the level of CEA and E-cadherin expression in metastatic and non-metastatic sample groups.

Method: The present study is descriptive with a quantitative approach using ANOVA one-way, unpaired t-test, and Pearson correlation analysis for the measurement and comparison of the CEA level and relative gene expression value from the reverse transcription-quantitative polymerase chain reaction analysis.

Results: The obtained results suggested increasing CEA level and decreasing E-cadherin expression on the metastatic sample. Statistically, E-cadherin proven to show a negative r value or correlation value of CEA, even though it has a significant *P*-value. In other parameters, alanine transaminase and aspartate aminotransferase indicated a positive r value and a significant *P*-value.

Conclusion: These findings indicated the potential clinical benefit of E-cadherin in detecting tumor progressivity, supported by other significant parameters, such as alanine transaminase and aspartate aminotransferase. Furthermore, E-cadherin was found beneficial in diagnosing the colorectal carcinoma with liver metastatis. Nonetheless, further research is needed to determine the role of E-cadherin regulation in colorectal cancer metastatis.

Keywords: Colorectal neoplasms, Neoplasm metastatic, Non-metastatic, Carcinoembryonic antigen, E-Cadherin

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Introduction

Colorectal carcinoma (CRC) is an epithelial malignant tumor in the colon and rectum area.[1] In 2020, World Health Organization (WHO) reported that CRC is the most prevalent cancer diagnosed in males (ranked third with 10%) and females (ranked fourth with 9.4%) out of the total global cancer cases.[2] Meanwhile, in the same year, The International Agency for Research on Cancer also reported that CRC is ranked as the third cause of death. The incidents and death rate of CRC vary greatly depending on the geographical location. Around 55% of colorectal cases are recorded in industrial countries,[3] while the highest death rate is found in developing countries.[4] Siegel et al. (2020) explain that around 147,950 cases were recorded in the United States of America, with 53,200 death cases, estimated as 9% of the total death rate of cancer mortality.[5] In 2020, Global Cancer Observatory also reported 34,189 (8.6%) cases of CRC in Indonesia, out of a total of 396,914 and 12,425 cancer cases in males (ranked second) and females (ranked fourth), respectively.

The causes and pathogenesis of CRC are correlated with genetic and environmental factors.[6] The mutation accumulation has a role in the cell growth regulation and differentiation, which affects the methylation of the tumor suppressor gene, inactivating and prompting neoplasia.[7] Additionally, the environment contributes to the increase in CRC. Consumption of alcohol, nicotine, processed meat, lack of physical activities, heredity, and obesity enhances the risks of CRC, as well as the increase in age and gender.[8,9,10]

One of the markers capable of detecting CRC is carcinoembryonic antigen (CEA) within a serum. CEA is a glycoprotein extracted from CRC tissue.[11,12] It is an oncofetal antigen produced by epithelial tumor cells and endodermal derived in the gastrointestinal tract.[13] Detection with CEA is considered safe, non-invasive, inexpensive, and easy to perform.[14,15] CEA is also used in laboratory tests, as the biomarker for the screening, diagnosis, and treatment.[11] Moreover, Nadeem, M. (2018) reported that around 74% of patients present increasing CEA levels, following the advancement of CRC stages.[12]

Generally, the primary process of distant metastatic is the transmission of epithelial-mesenchyme, with the tumor cell impairing the normal epithelial structure and substituting it with a fibroblast-like phenotype.[13] This epithelial-

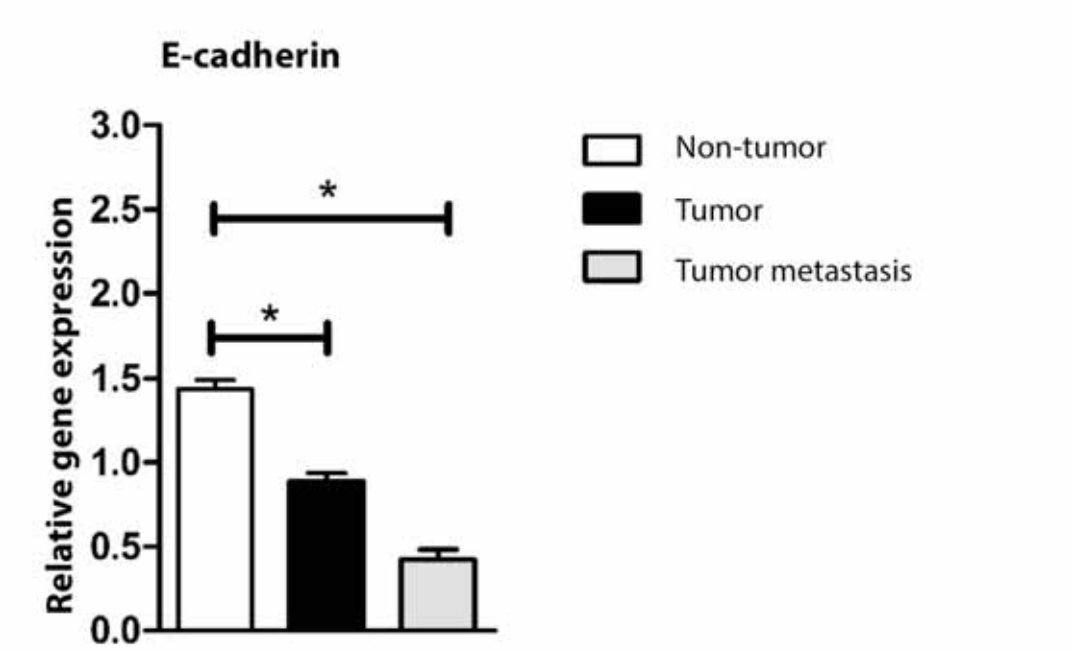

Figure 1. This figure illustrates the E-cadherin gene expression levels in different tissues of the CRC patients.
* Significant with $P \leq 0.0$ with ANOVA one way test; CRC: Colorectal cancer

mesenchyme results in decreasing the adhesion between cells, stronger infiltration power, and increasing cell apoptotic resistance.[14] It is correlated with the zinc-finger factors, functioning as transcription factors, such as Snail1 and ZEB1/2.[15,16] These two genes also affect the E-cadherin expression in CRC. Kaszak et al. (2020) explained that the E-cadherin gene possesses an essential role in tissue homeostasis as it is liable for cell adhesion during embryogenesis, tissue morphogenesis, differentiation, and carcinogenesis.[17] The instability and disfunction during its regulation results in E-cadherin triggering the tumor development.[18]

Based on this background, the CRC that has been developed into distant metastatic activates the epithelial-mesenchymal transition process. Therefore, we conducted the present work as it is essential to investigates and compare the level of CEA and E-cadherin gene expression on CRC before and after the liver metastatic; thus, new diagnostic and treatment approaches for CRC would be identified.

Materials and Method

Research samples

This descriptive study used a quantitative approach. Meanwhile, a quantitative approach was utilized in measuring the CEA level and E-cadherin gene expression on 45 samples, consisting of 20 CRC non-metastatic colon tissues, 12 CRC hepatic metastatic colon tissues, and 13 normal colon tissues from different patients diagnosed with CRC. The surgeon team of Dr. Sardjito Central General Hospital, Yogyakarta, collected the CRC tissue samples. We conducted this study from December 2021 to February 2022. Subsequently, data measurement was carried out in the Central Laboratory of Advanced Minerals and Materials, Universitas Negeri Malang, and Central Laboratory of Biological Sciences, Universitas Brawijaya.

Human rights and informed consent

This study attained ethical clearance from the Health Research Ethics Committee of Dr. Sardjito Central General Hospital, Yogyakarta, under the number KE/FK/0938/EC/2021. The samples were obtained from the patients with confirmed CRC. This study applied a purposive sampling method, with the inclusion criteria being only the colorectal patients who had undergone surgery, either with or without liver metastases. Written informed consent was received form all the patients before study. The exclusion criteria, on the other hand, were having agreed to participate in other studies, having peritoneal metastases, not having received

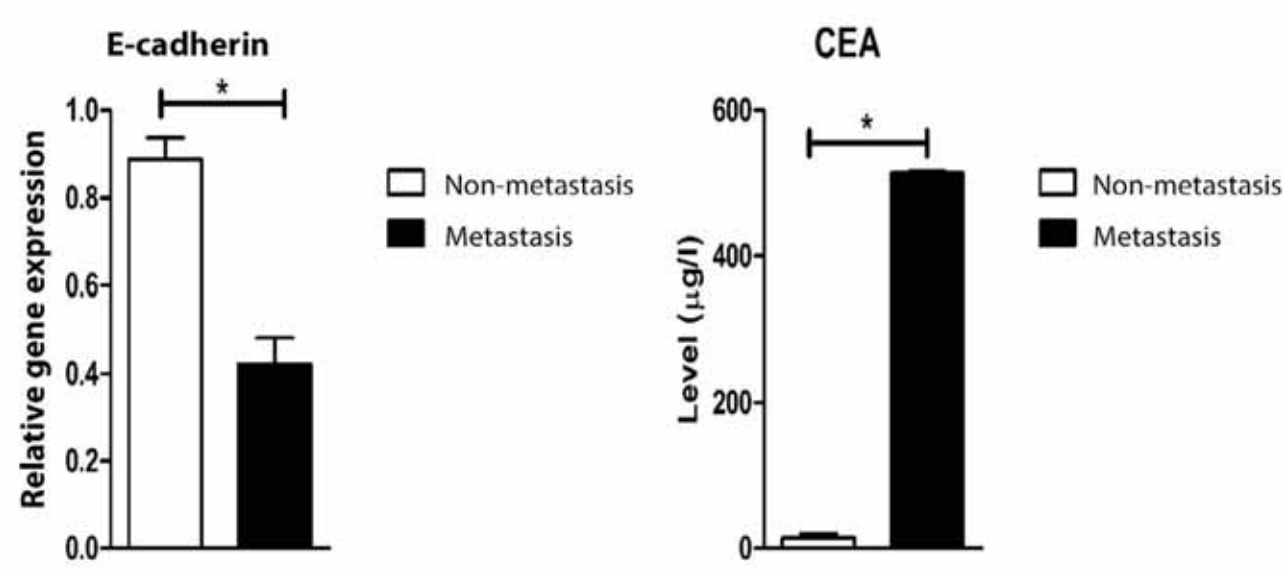

Figure 2. This figure illustrates E-cadherin and CEA profile in the non-metastatic and metastatic CRC patients.
*Significant with $P \leq 0.05$ with ANOVA one way test; CRC: Colorectal cancer; CEA: Carcinoembryonic antigen

preoperative chemotherapy or radiotherapy, not meeting the inclusion criteria.

Measurement of baseline characteristic parameters

Anthropometric data were measured for all the study subjects. Briefly, the study subjects were rested for 15 minutes, after which the patient's blood pressure was measured on the right arm in a sitting position (normal). All the patient parameters/baseline characteristics were measured with standard clinical methods according to the basic anthropometric examination protocol. The baseline characteristic data included age, body mass index (BMI), systolic blood pressure (SBP), diastolic blood pressure (DBP), white blood cells (WBC), red blood cells (RBC), blood urea nitrogen (BUN), platelet count (PLT), alanine aminotransferase (ALT), aspartate aminotransferase (AST), CEA. In addition, we checked the pathological grading (under the categories of well, moderate, and poor) and tumor location.

Gene expression measurement

We carried out a quantitative gene expression test by isolating the total RNA from our patients' tumor samples, which was tested via real-time quantitative polymerase chain reaction (RT-qPCR). The sample was a fresh tumor containing tumor tissue (diagnosed as colonic adenocarcinoma) at Dr. Sardjito Hospital, Yogyakarta. To this end, 100 mg of fresh tumor samples were weighed, following which the sample was ready for the total RNA isolation process using kit TRISURETM / Qiazol (BIOLINE, UK). The RNA samples were then stored for RT-PCR at -20°C in the refrigerator. Afterwards, via reverse transcription PCR, we carried out a conversion from RNA to cDNA using a reverse transcription kit with ReverTra Ace® qPCR RT Master Mix with gDNA Remover (TOYOBO, Japan). Then, we utilized RT-PCR with Analytik Jena qTower Machine, using mixed solution following the formula from SensiFAST SYBR Green No-ROX kit (BIOLINE, UK). The primers for E-cadherin and β-actin used in our study included: E-cadherin as a target gene in this study, forward 5′ AAAGGCCCATTTCC-TAAAAACCT 3′ and reverse 5′ TGCGTTCTCTATCCAGAGGCT 3′.[19] The housekeeping gene using β-actin, forward 5'-CATGTACGTTGCTATCCAGG -3′ and reverse 5′- CTCCTT AATGTCACGCACGAT -3′.[20] The RT-qPCR temperature set by following the Kit SYBR Green 2-step cycling as follows: 1 cycle at 95°C for 2 minutes for polymerase activation, followed by 40 cycles at 95°C for 10 seconds for the denaturation step and with the Tm primary temperature set at 30 seconds for the annealing/extension step.

CEA level measurement

Body surface area (BSA) blocked bead–antibody complexes, and labeled primary antibodies and fluorescence-labeled secondary antibodies were added to the centrifuge microfluidic device and incubated at room temperature for 2 hours. The clinical serum samples, collected and provided by the affiliated Hospital of Dr. Sardjito Yogyakarta, Indonesia, from both cancer metastatic and non-metastatic individuals, were then added to the centrifuge chip and spun at 2500 rpm for 2.5 min. After obtaining the fluorescence images using the fluorescence microscope and processing with the ImageJ software, we used the standard concentration curve for obtaining the experimental CEA concentrations.

Data analysis technique

Measuring the mRNA level, we investigated the expression of the E-cadherin gene, while the RT-PCR resulted in the cycle threshold (Ct). The mRNA level (relative gene expression) was analyzed using Relative Quantification and the 2ºCt formula. The RT-PCR results were then analyzed using JMP.6 software (Chicago, IL; North Carolina, USA). The data normality was also investigated with the Kolmogorov-Smirnov test. Subsequently to compare the data from each group (between non-metastatic and metastatic), the data was analyzed with Anova-One Way. We identified the E-cadherin levels between the sample groups. Then, we compared the level of relative gene expression of E-cadherin and CEA via t-test and univariate correlation using Pearson correlation. The significance level of 5% or $P \leq 0.05$ indicated a significant statistical difference.

Table 1. Baseline characteristics of the study population from different groups

Parameters	Groups	
	Non-metastatic (n = 20)	Metastatic (n = 12)
Age (yrs)	54.50 ± 3.12	57.76 ± 1.98
BMI (kg/m2)	23.41 ± 1.19	19.40 ± 0.48*
SBP (mmHg)	123.15 ± 2.43	120.67 ± 2.79
DBP (mmHg)	78.10 ± 1.99	77.43 ± 1.99
Hemoglobin (g/dL)	11.38 ± 0.47	11.56 ± 0.35
Haematocrit (%)	34.91 ± 1.37	35.28 ± 1.01
WBC (103/µl)	8.77 ± 0.84	10.76 ± 0.88
RBC (106/µl)	4.38 ± 0.12	4.19 ± 0.10
Blood Glucose (mg/dL)	120.80 ± 10.28	142.33 ± 10.55*
Albumin (g/L)	3.63 ± 0.16	3.14 ± 0.16
BUN (mg/dL)	12.69 ± 1.49	14.79 ± 1.57
Creatinine (mg/dL)	0.91 ± 0.71	0.92 ± 0.08
PLT (103/?l)	284.10 ± 21.33	352.62 ± 20.92
ALT (U/L)	11.65 ± 1.74	48.81 ± 2.83*
AST (U/L)	21.35 ± 4.51	56.29 ± 2.83*
CEA (µg/L)	14.36 ± 5.51	513.91 ± 2.26*
Pathological Grading		
Well, moderate	18	6
Poor	2	6
Tumor location		
Colon	11	4
Rectum	9	8

BMI: Body mass index; SBP: Systolic blood pressure; DBP: Diastolic blood pressure; WBC: White blood cells; RBC: Red blood cells; BUN: Blood urea nitrogen; PLT: Platelet count; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; CEA: Carcinoembryonic antigen; Independent samples t-test was used to compare the differences among groups. Data are presented as mean ± SEM. * Significant value of each parameter compared to non-metastatic group ($P \leq 0.05$) by unpaired t-test.

Results

This study was carried out on 45 samples, consisting of 20 CRC non-metastatic tissues, 12 metastatic CRC/liver metastatic tissues, and 13 normal colon tissues from different patients diagnosed with CRC. The results of the examination revealed that 20 non-metastatic and 12 metastatic CRC patients experiencing a significant increase in CEA level were also observed on the metastatic CRC samples. The data in table 1 show the serology test ANOVA results (baseline characteristic parameters) of the CRC metastatic and non-metastatic groups. These data suggested significant differences in terms of BMI, fasting blood sugar, as well as the level of ALT, AST, and CEA between the metastatic and non-metastatic groups. Based on the results of the Pearson correlation test represented in table 2, it is known that the ALT, AST, and CEA parameters had significant *P* -values. Nonetheless, the CEA levels had a negative r value (correlation). Similarly, the sex parameters, BMI, RBC, albumin, creatinine, and PLT had negative r values as well.

The results of Ct values, calculated using 2ºCt, represented the relative expression of E-cadherin and CEA and were visualized using GraphPad Prism 5 (Figures 1 and 2). According to the results, the lowest E-cadherin expression belonged to the group of tumors that had liver metastatic compared with the tumor group without metastatic and non-tumor group (Figure 1). We observed the increasing level of CEA (Figure 2, right side) and lower E-cadherin expression (Figure 2, left side) in the liver metastatic group in comparison with the non-metastatic group, meaning they were inversely proportional.

Discussion

In this study, we used the level of CEA and E-cadherin expression as the marker for diagnosing the incidence of CRC. This study revealed that CEA levels increased drastically in the group of metastatic patients (Figure 2, right

Table 2. Univariate correlations of E-cadherin in all the participants

Parameters	E-cadherin	
	R	*P*-value
Sex	-0.156	0.394
Age (yrs)	0.285	0.114
BMI (kg/m^2)	-0.182	0.318
SBP (mmHg)	0.222	0.222
DBP (mmHg)	0.062	0.738
Hemoglobin (g/dL)	0.091	0.620
Haematocrit (%)	0.015	0.937
WBC (103/µl)	0.192	0.292
RBC (106/µl)	-0.207	0.255
Fasting glucose level (mg/dL)	0.012	0.949
Albumin (g/L)	-0.163	0.372
BUN (mg/dL)	0.045	0.805
Creatinine (mg/dL)	-0.069	0.707
PLT (103/µl)	-0.039	0.833
ALT (U/L)	0.761	0.000*
AST (U/L)	0.742	0.000*
CEA (µg/L)	-0.359	0.043*

* Significant with $P \leq 0.05$ with Pearson product-moment correlation test; BMI: Body mass index; SBP: Systolic blood pressure; DBP: Diastolic blood pressure; WBC: White blood cells; RBC: Red blood cells; BUN: Blood urea nitrogen; PLT: Platelet count; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; CEA: Carcinoembryonic antigen

side). This is in line with the results of Saito et al. (2016) who reported increasing CEA concentration levels in CRC patients.[19] CEA adheres to the cell membrane through a glycosylphosphatidylinositol bond and can be released as solute formed by phospholipase C or phospholipase D.[19] CEA presumably serves as an adhesion molecule contributing to cancer invasion and metastatic.[20] Su et al. (2012) also explained that CEA modulates the adhesion between collagen and epithelium cells in the colon.[21] Increased concentrations of CEA levels can also be caused by environmental factors, namely obesity, lack of physical activity, alcohol consumption, and nicotine.[22] The increased risk of CRC incidence can also be caused by age and gender.

The results showed different E-cadherin expressions in the non-metastatic and metastatic CRC sample groups, with significantly decreasing E-cadherin expression in the metastatic CRC sample group. E-cadherin is associated with the tumor suppressor gene, while decreased expression is associated with poor prognosis and metastatic.[23] Downregulation of E-cadherin is associated with poor differentiation, vascular invasion, metastatic, and advanced stage of cancer. Moreover, decreased expression of E-cadherin gene facilitates the epithelial-mesenchymal transition (EMT), the indicator of epithelial cells transition into mesenchymal cells, under physiological and pathological situations.[24] Therefore, EMT is presumed to be one of the primary mechanisms that determine the invasive spread and metastatic of cancer cells.[25] CRC that undergoes EMT can invade and metastasize to other organs, such as the liver, through direct diffusion as well as blood and lymphatic circulation.[26] Decreasing E-cadherin expression on the cell membrane weakens the interaction between cells, inhibits the activation of transcription factors (Snail, Slug, Twist dan ZEB-1), and results in EMT.[27] Sterneck et al. (2020) also mentioned that the Snail family gene is the transcription factor suppressing the E-cadherin gene.[28] E-cadherin depends on Ca (calcium) for efficient adhesion between cells; when calcium deficiency occurs, it causes instability of cell structure, increases EMT, and facilitates gaining mobility and invasion for cells.[24,29]

The findings herein also suggested the increase in ALT and AST, the markers of liver fibrosis. ALT is an enzyme that changes protein into the energy used by the liver cells, while AST is an

enzyme with a role in the metabolism of amino acids.[30] ALT can be found in the liver, kidneys, heart, muscles, and pancreas, whereas AST is found in RBCs, liver, heart, muscle tissue, pancreas, and kidneys. ALT and AST levels are used to determine the level of hepatocellular damage. When hepatocellular damage occurs, liver cells release these enzymes into the bloodstream, which raises levels of ALT-AST.[31] The constant increase in these two enzymes escalates the risk of CRC.[31,32]

The results of BMI data showed a significant difference with the level of relative gene expression of E-cadherin. People with excess BMI are classified as obese and have high lipoprotein levels.[33] Obesity increases the risk of diabetes mellitus 2, cardiovascular diseases, and some cancers.[34] High triglyceride levels cause fat accumulation in liver tissue, which is known as fatty liver disease and can cause cancer. Accumulation of free fatty acids triggers the production of oxidants, thereby inhibiting lipogenesis and causing inhibition of triacylglycerol clearance. Triacylglycerol causes an increase in blood triglyceride levels (hypertriglyceridemia), causing cardiovascular events.[35]

The fasting glucose levels showed a significant difference. The insulin system or IGFs (Insulin-like growth factors) are involved in the pathogenesis of colon cancer. IGF1 and IGF-1R overexpression levels were detected in CRC and activated Src, thereby leading to increased proliferation and migration (metastatic) of colon cancer.[36,37] Insulin binds to the IGF-1 receptor, thus increasing cell proliferation and the occurrence of colon cancer.[38–41] The proliferation of these cells occurs through the activation of the phosphoinositide 3-kinase (PI3K)/Akt signaling pathway. This activation plays a role in the transformation and growth of colon cancer cells.

Based on the obtained results, an increase in CEA level along with a significant decrease in E-cadherin were observed in the metastatic sample group. Furthermore, an increase in ALT and AST was also found in the same group. Therefore, the results of this study can be used as the baseline data for further research development. These findings are expected to help the identification of preventive methods and treatments for CRC patients. Nevertheless, this study has certain limitations due to the limited sample size and areas of sample; therefore, further research is needed to cover other areas.

Conclusion

The increase in CEA level and a significant decrease in E-cadherin were observed in the metastatic sample group. Additionally, a significant increase in ALT and AST was found in the same metastatic group. This indicates that the E-cadherin and CEA are capable of becoming candidate biomarkers, for CRC in particular, in the future. E-cadherin expressed in the samples can also be coupled with the other parameters to determine the expression pathway. This can help the improvement of diagnostic and prognostic information for patients and further research.

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Conflict of Interest

None declared.

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Vitamin D Serum Levels in Oral Lichen Planus and Oral Cancer Patients

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Abstract

Background: This study aimed to evaluate serum vitamin D levels in patients with oral lichen planus (OLP) and oral squamous cell carcinoma (OSCC) in comparison to healthy controls in an Iranian population.

Method: A cross-sectional study was conducted, which included 69 patients with OLP, 40 patients with OSCC, and 60 healthy controls. Serum vitamin D levels were measured using the ELISA method. The data were analyzed using Mann-Whitney and T-tests, and statistical significance was set at $P < 0.05$.

Results: The study found that 17.9% of OLP patients, 27.25% of OSCC patients, and 25% of the control group had normal vitamin D levels. The mean vitamin D level in OLP patients (17.00 ± 14.16 ng/mL) was significantly lower than that in the control group (22.99 ± 14.46 ng/mL) ($P = 0.003$). However, in OSCC patients, the mean vitamin D level (24.63 ± 16.19 ng/mL) was not significantly different from that of the control group.

Conclusion: The study revealed a high rate of vitamin D deficiency and insufficiency in OLP, OSCC, and control group patients. Vitamin D deficiency was more common in patients with OLP. Vitamin D deficiency may potentially increase the risk of OLP and OSCC development and progression.

Keywords: Neoplasms, Lichen Planus, Oral, Vitamin D, Serum

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Introduction

Oral lichen planus (OLP) is a common muco-cutaneous disorder that is considered a premalignant condition with an unknown etiology. Although some studies have shown the role of the immune system and some predisposing factors in its pathogenesis1, the exact cause remains unclear. Studies have

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reported that 1%-2% of OLP lesions transform into oral squamous cell carcinoma (OSCC), and the frequency of malignant transformation of OLP is between 0.4% and 5%.[2]

OSCC accounts for more than 90% of all oral cancers, with tobacco usage being the principal risk factor. Other risk factors include socioeconomic status, genetic predisposition, and so forth. A diversity of endogenous and exogenous stimuli leads to a complex series of molecular changes that participate in cancer development.[3]

1, 25-dihydroxy vitamin D3 [1,25(OH)2D3] is the active form of vitamin D3, a fat-soluble vitamin that is synthesized in the skin from exposure to sunlight by UV light-mediated modifications. This vitamin is involved in the pathogenesis of several autoimmune, inflammatory, and cancerous diseases. In several studies, vitamin D has shown antiproliferative and pro-differentiation properties in a number of normal and cancer cells.[4] It affects and regulates the immune system cells and acts as an anti-inflammatory agent. Moreover, vitamin D contributes to the induction of apoptosis, inhibition of invasiveness, and angiogenesis in several human cancers[4] and has shown growth-inhibitory effects on oral cancer cell lines.[5] Additionally, calcitriol has been found to enhance the effectiveness of cytostatic chemotherapy to induce apoptosis in OSCC cells.[6]

Vitamin D deficiency has been correlated with some autoimmune diseases such as rheumatoid arthritis, systemic lupus erythematosus, and multiple sclerosis, and it has shown potential therapeutic effects on some autoimmune diseases.[7] Several studies that have evaluated vitamin D serum levels in patients with OLP as well as OSCC have reported controversial results. Guta et al.[8] showed that vitamin D deficiency in OLP patients was lower than in healthy subjects. Afzal et al.[9] showed that lower vitamin D levels decreased in OSCC patients, whereas Aremd et al.[10] found no change.

Although there is great interest in investigating the anticancer properties of vitamin D, few studies have explored these effects in oral precancerous lesions and oral cancer in a limited number of patients with controversial results. Other studies used vitamin D in the treatment of OLP without strong evidence of the vitamin status in patients and reported favorable results.[8] Due to the high prevalence of OLP and OSCC, it is crucial to determine the role of vitamin D in their progress and pathogenesis. The aim of the present study was to evaluate the vitamin D status in a group of OLP and OSCC patients.

Table 1. Baseline data of all groups

Groups	Mean Age ± SD	M:F
OLP (69)	55.33 ± 11.60	21:48
OSCC (40)	62.60 ± 15.53	26:14
Control (60)	51.01 ± 13.74	18:42
Control (60)	51.01 ± 13.74	18:42

OLP: Oral lichen planus; OSCC: Oral squamous cell carcinoma; SD: Standard deviation; M: Male; F: Female

Method and Materials

This cross-sectional study was approved by the Medical Ethics Committee of Shiraz University of Medical Science (IR.SUMS.Dental.REC 1399.016).

In this study, 173 serum samples were collected during 2018-2020. Specifically, there were 70 samples from patients with OLP, 43 samples from patients with OSCC, and 60 serum samples from healthy subjects. These patients were selected from individuals who referred to the department of oral medicine at Shiraz Dental School. All patients met the clinical and histopathological criteria for OLP and OSCC. Excluded patients were those who had taken complementary and vitamin D supplements within the past two months, as well as those with diseases that alter vitamin D serum levels, such as thyroid or parathyroid diseases and hyperparathyroidism, the presence of other cancers, systemic or inflammatory diseases, and cases with a history of previous anticancer treatment and disease recurrence. The healthy subjects did not have any of the inclusion or exclusion criteria. Informed consent was obtained from all study participants.

Serum vitamin D levels were measured by the ELISA test using an antivitamin D3 ELISA kit (Monobind, cat #7725-300) according to the

Table 2. Vitamin D levels in different clinical presentations of patients

Clinical presentation	Percentage	Mean of Vitamin D	*P*-value vs. control
OLP			
Keratotic	68	17.42 ± 15.55	0.01
Non-keratotic	32	16.29 ± 14.49	0.03
Stages of OSCC			
Stage 1	17.5	33.20 ± 19.58	0.21
Stage 2	32.5	22.33 ± 13.54	0.92
Stage 3	25	30.10 ± 19.30	0.29
Stage 4	25	16.13 ± 9.22	0.20

OLP: Oral lichen planus; OSCC: Oral squamous cell carcinoma

manufacturer's instructions. A 2CC blood sample was taken from each study participant and kept at -20°C in sterile tubes. Data were analyzed using the Mann-Whitney U, Kruskal-Wallis, and Pearson Chi-Square tests by SPSS 17. Statistical significance was set at $P < 0.05$.

Results

40 OSCC patients, 69 OLP patients, and 60 controls were enrolled in the study. Four cases with outlier data were removed. The mean age ± standard deviation (SD) of participants was 62.60 ± 15.53 for OSCC, 55.33 ± 11 for OLP patients, and 51.01 ± 13.74 for the controls. Baseline data of all study groups are illustrated in table 1.

Most of the OLP patients had the erosive and reticular type of OLP, and most of the OSCC patients were in stage 2 of the disease; both groups were not significantly different (Table 2).

Table 3 shows the mean vitamin D levels in all groups. The mean vitamin D levels in OLP patients (17.00 ± 14.16 ng/mL) were significantly lower than those of the control group (22.99 ± 14.46 ng/mL) by Mann-Whitney test ($P = 0.003$). In the OLP group, 70% of patients suffered from vitamin D insufficiency, which was significantly lower than the vitamin D insufficiency in the control group (46.7%) (chi-square test, $P = 0.04$). The mean vitamin D levels in females with OLP lesions were significantly lower than those in the females of the control group (Mann-Whitney test, $P = 0.004$), but this difference was not found in males ($P = 0.31$). Details are illustrated in table 4. Moreover, the Mann-Whitney test showed that no significant difference existed between the mean vitamin D levels in males with OLP lesions and the control group (17.81 ± 24.45 ng/mL) in comparison with the females (20.25 ± 16.58 ng/mL) ($P = 0.96$).

The mean vitamin D levels in both erosive (16.29 ± 14.49 ng/mL) and reticular (17.42 ± 15.55 ng/mL) types of OLP were lower than those of the control group (Mann-Whitney test, $P = 0.009$ and $P = 0.03$, respectively).

The mean of vitamin D in OSCC patients (23.8 ± 16.19 ng/mL) was not significantly different from that of the control group based on the Mann-Whitney test ($P = 0.78$). Therefore, 51.1% of OSCC patients had vitamin D insufficiency, and 23.2% had deficiency, which was not significantly different from vitamin D insufficiency (46.7%) and deficiency (28.3%) in the control group (chi-square test, $P = 0.64$).

The Mann-Whitney test revealed that the mean vitamin D levels in males and females with OSCC were not significantly different from those in healthy males and females. Moreover, the mean vitamin D levels in males with OSCC and the control group (24.51 ± 16.76 ng/mL) were not significantly different from females (21.19 ± 15.33 ng/mL) ($P = 0.88$). Details are shown in table 4. Furthermore, the vitamin D levels in the control group, OLP and OSCC patients were not related to their age (Pearson correlation, $P > 0.05$).

Discussion

In this study, the serum levels of vitamin D were evaluated in a group of patients with OLP and OSCC and compared with those of a group of healthy individuals. The findings showed a high rate of vitamin D deficiency and insufficiency

Table 3. Vitamin D mean and status in all groups

D3 level	OSCC	OLP	Control	*P*- value vs. control	
				OSCC	OLP
Mean	24.63 ± 16.19	17.00 ± 14.16	22.99 ± 14.46	0.04	0.77
Insufficiency (%)	52.5	69	46.7	0.07	0.91
Deficiency (%)	20	12.9	28.3	0.69	0.96
Sufficiency (%)	27.5	17	25	0.93	0.76

OLP: Oral lichen planus; OSCC: Oral squamous cell carcinoma

in all study groups. Vitamin D deficiency was more frequent in patients with OLP.

The study findings revealed that vitamin D deficiency was prevalent in OLP and OSCC patients, as well as in the control group. 72.5% of OSCC patients, 82.9% of OLP patients, and 75% of the control group suffered from vitamin D deficiency or insufficiency. This finding confirms the high prevalence of vitamin D deficiency in the Iranian population, which is a major health problem in Iran, especially in females.[11] Cultural and social taboos that affect life-style patterns, such as dressing habits, which limit sun exposure in females, may be a reason for vitamin D deficiency in Iran.[12]

The levels of vitamin D, as well as vitamin D sufficiency, were significantly lower in the OLP group compared with the control group. In particular, most of the OLP females and males suffered from low amounts of vitamin D, with females having significantly lower levels than the disease-free controls. Some researchers have reported similar findings.[8, 13, 14] Gupta et al. have stated that urban people, vegetarians, and middle/lower-middle socio-economic classes are more likely to suffer from vitamin D deficiency,[8] although controversial results have been reported. It has been suggested that vitamin D plays a vital role in the initiation and severity of OLP through its regulatory effect on the immune system.[8] Vitamin D deficiency results in a decrease in Th2 cell counts compared with other T cells that regulate inflammatory pathways.[15] Zhao et al.[16] have demonstrated that lipopolysaccharides downregulate the VDR expression by the keratinocytes in OLP tissue compared with normal oral mucosa. This function is dependent on the tumor necrosis factor-alpha (TNFα)-miR-346 pathway, and vitamin D/VDR could play a protective role in the integrity of oral mucosa.

In our study, mean vitamin D levels in both erosive/atrophic and reticular types of OLP were lower than control patients, which is consistent with some previous reports.[8, 13] Moreover, erosive lichen planus is associated with severe pain and burning that can be related to vitamin D deficiency in these patients.[8] Ahmed et al. have shown that vitamin D deficiency is not only related to the development of OLP but also related to the symptoms and types of OLP.[13] Studies have shown the role of vitamin D in autoimmune diseases such as multiple sclerosis, rheumatoid arthritis, systemic lupus erythematosus, inflammatory bowel disease, asthma, and infectious diseases.[17, 18] Lack of vitamin D has been found to increase the cytotoxic activity of cells.[19]

Our findings regarding the high incidence of vitamin deficiency in OSCC patients were in agreement with previous studies. Grimm et al.[20] reported that among their 42 OSCC patients, 100% of them had moderate to severe vitamin D deficiency. Anand et al.[21] reported 76.4% deficiency among their patients, and another study reported 65% vitamin D deficiency and insufficiency in the OSCC group.[19] In this study, no significant difference existed between vitamin D levels in the OSCC and the control group. Arem's study also showed no association between serum 25(OH) D and the risk of head and neck cancers.[10] They reported a mean vitamin D level of about 31 in their study. Their patients were male smokers from the white population. In contrast, data from other studies[19, 22, 23] have shown a significant difference between the cancer and control groups. The mean vitamin D levels in our OSCC group were 24.6 ng/mL, which is in agreement with 22 and 20.4 ng/mL in previous studies.[19, 23] These results indicate that the lack

Table 4. Mean of vitamin D levels based on patients' gender

	OLP	OSCC	Control	*P*-value vs. control	
				OLP	OSCC
Female	17.16	28.93	29.68	0.004	0.59
Male	17.81	22.31	20.19	0.30	0.88

OLP: Oral lichen planus; OSCC: Oral squamous cell carcinoma

of association between vitamin deficiency and SCC development in the present study is mostly related to a high rate of vitamin deficiency among the control group. In our study, 25% of the control group had normal vitamin D levels, and this rate was 46.90% and 38% in the aforementioned studies.[10, 23]

Vitamin D and its metabolites reduce the incidence of various cancers by inhibiting tumor angiogenesis, stimulating mutual adherence of cells, and enhancing intercellular communication, thereby strengthening the inhibition of cellular proliferation.[21, 24]

In our OSCC group, the mean vitamin D levels were lower in the last stage, with no significant difference with other disease stages, which was probably due to our limited sample size of each stage. Udeabor et al.[23] have shown that vitamin D levels were decreased in the late stages of SCC. This could be because of disease severity and complications during this stage. One study has shown that in patients with different head and neck tumors, higher vitamin D serum levels were associated with better survival and progression-free survival.[25] Bochen et al.[22] found a significant association between higher vitamin D serum levels with a negative lymph node status and a possible inhibitory effect of Vitamin D on tumor cell metastasis. They also showed that vitamin D status was related to the patients' survival rate. In patients with advanced cancer stages, it has been shown that vitamin supplementation reduced therapy-related toxicities and improved the quality of life of patients.[13, 22]

A few limitations were presented in our study, similar to most of the previous research on vitamin D levels, including the lack of data about the duration of previous exposure to sunlight, BMI, number of pregnancies, and parathyroid hormone and calcium serum levels. Moreover, the final period of the study coincided with the COVID-19 pandemic, which resulted in increased vitamin D consumption among the entire population, and this factor prevented an exact gender match between the cancer and other groups.

Conclusion

In this study, there was a high rate of vitamin D deficiency and insufficiency in patients with OLP and OSCC, as well as in the control group. The mean serum levels of vitamin D in patients with OLP were significantly lower than in healthy subjects; however, the difference was not significant between OSCC patients and the control group. This could be mostly attributed to the high rate of vitamin deficiency in the control group. The results of the present study further corroborate the assertion that vitamin D deficiency may be a potential risk factor in the development and progression of OLP and OSCC. As with patients who have other immunologic disorders, vitamin D deficiency should be considered in patients with OLP and OSCC, regardless of the site and type of OLP and OSCC. Given the high attention paid to vitamin D consumption during the Coronavirus disease 2019 (Covid-19) pandemic period, further studies are suggested to compare the incidence of these disorders before and after vitamin therapy.

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Conflict of Interest

None declared.

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The Effect of Bortezomib Regimen on Multiple Myeloma Patients Infected with COVID-19

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Abstract

Background: Patients with multiple myeloma (MM) have compromised immune systems due to the nature of the malignancy and anticancer treatments. This study aims to report the effects of Bortezomib-containing chemotherapy regimens on the severity and mortality of MM patients infected with severe acute respiratory syndrome coronavirus 2 (SARS-COV-2).

Method: This retrospective cohort study enrolled MM patients presenting with coronavirus disease 2019 (COVID-19) infection referred to Omid Hospital. Patients were divided into two groups based on whether they received any chemotherapy regimens containing Bortezomib within the last 90 days of admission or not. Clinical and laboratory characteristics, severity, and outcomes of both groups were reported and compared.

Results: Among 48 patients with MM diagnosed with COVID-19 (63% male; median age 66), 33 received chemotherapy. The most common symptoms were fever, cough, and dyspnea, and there was no significant difference between the groups. Only D-dimer had a significant difference in laboratory tests ($P = 0.03$) and was higher in the chemotherapy group. There was no significant relationship between chemotherapy and severity (risk ratio (RR) = 1.17; 95% confidence interval (CI): 0.37 to 3.71; $P = 0.79$) or chemotherapy and mortality (RR= 1.00; 95% CI: 0.39 to 2.61; $P = 0.99$), even after adjusting for baseline C-reactive protein and white blood cell counts.

Conclusion: Our study showed that receiving Bortezomib-containing chemotherapy regimens did not worsen the symptoms and prognosis of MM patients infected with COVID-19. However, further studies with larger sample sizes and longer follow-up times are needed to provide better evidence on this subject.

Keywords: COVID-19, Cancer, Hematologic neoplasms, Multiple myeloma, Bortezomib

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Introduction

Since the end of 2019, the emerging pandemic caused by the severe acute respiratory syndrome coronavirus (SARS-CoV-2) has raised concerns for cancer patients due to their impaired immunity and vulnerability to infections.[1] It is now known that cancer patients are at a higher risk of developing severe forms of coronavirus disease 2019 (COVID-19) and experiencing higher mortality rates, with the risk varying among different cancer types.[2, 3] Patients with hematologic malignancies are more vulnerable to COVID-19 than those with solid malignancies.[4] This increased risk poses a challenge for oncologists on whether to continue cancer treatments or not. Although vaccination reduces mortality and hospital admission rates among the general population and cancer patients, breakthrough infections have been reported in cancer patients,[5] with recent studies showing that the breakthrough infection rate is higher in cancer patients than in the general population.[6] This suggests that even after the pandemic, we may encounter cancer patients infected with COVID-19, and there is a need for evidence regarding treatments during this period.

Several studies have evaluated the effect of chemotherapy on cancer patients, resulting in systematic reviews and meta-analyses. However, there is inconsistency between the results. For instance, a meta-analysis of 16 studies by Yekeduz et al. concluded that chemotherapy within the last 30 days of diagnosis does not affect the severity, but increases the mortality rate of cancer patients.[7] In contrast, two other systematic reviews and meta-analyses by Liu et al. and Lin et al. showed that mortality has no significant difference between cancer patients under active treatment and cancer patients who are not.[8, 9] These contrasts highlight the need for studies on each specific chemotherapy regimen, as the type of cancer and chemotherapy drugs can affect the results.

Multiple myeloma (MM), the second most common hematological malignancy, is a proliferation of clonal B lymphoid cells that contribute to end-organ damage.[10] Patients with MM experience lower levels of humoral immunity due to their compromised production of proper immunoglobulins while facing over-secretion of monoclonal immunoglobulins. Moreover, the patients' cellular and innate immunity is also impaired.[11]

The standard therapy commonly used for active MM patients is triplet therapy, which is a combination of a proteasome inhibitor, an immunotherapy drug, and a corticosteroid.[12] Current evidence shows that proteasome inhibitors lead to a decreased cytotoxic T-cell response and susceptibility to viral infections.[13] Bortezomib, a proteasome inhibitor, is widely used in Iran to treat these patients.

To the best of our knowledge, no specific study has evaluated Bortezomib on cancer patients infected with COVID-19. Therefore, this study aims to assess the effects of chemotherapy regimens consisting of Bortezomib on the severity and mortality of MM patients infected with SARS-COV-2.

Material and Methods

Study design and participants

This is a retrospective cohort study of active MM patients with COVID-19 infection who were referred to Omid Hospital in Isfahan, Iran, from March 2020 to October 2021. Patients who had a positive SARS-CoV-2 test by polymerase chain reaction or lung involvement confirmed by chest computed tomography (CT) scan were included in the study. Exposure in this cohort study was defined as receiving any chemotherapy regimens that included Bortezomib within the last 90 days of admission. The control group did not receive Bortezomib within the last 90 days of admission due to Bortezomib-induced peripheral neuropathy. The decision to choose 90 days as the definition for recent chemotherapy was based on the article by Jee et al.[14] As the vaccination of cancer patients started in mid-2021 and receiving a vaccine can affect the outcomes, we excluded breakthrough infections from this study.

Data collection and follow-up

Demographic and clinical data, comorbidities such as diabetes, hypertension, cardiac disease,

Table 1. Patients' characteristics

	Total N = 48	Received chemotherapy (N = 33)	No chemotherapy (N = 15)	*P*
Demographics				
Gender (Male): n (%)	30 (63)	19 (58)	11 (73)	0.35
Age: Mean (SD)	66 (12)	65 (12)	67 (11)	0.64
Hospitalization length				
Median [Q1 – Q3]	7 [5 – 11]	7 [5 – 12]	7 [5 – 9]	0.69
ICU admission: n (%)	12 (25)	10 (30)	2 (13)	0.29
Blood products received: n (%)				
Packed cell	24 (50)	16 (48)	8 (53)	1.00
Fresh frozen plasma	11 (23)	6 (18)	5 (33)	0.28
Platelet	12 (25)	7 (21)	5 (33)	0.48
Symptoms of SARS-CoV-2 infection: n (%)				
Fever	22 (46)	14 (42)	8 (53)	0.54
Cough	15 (31)	11 (33)	4 (27)	0.75
Dyspnea	15 (31)	11 (33)	4 (27)	0.75
Weakness	10 (21)	7 (21)	3 (20)	1.00
Nausea	8 (17)	6 (18)	2 (13)	1.00
Lose olfactory or taste sense	3 (6)	3 (9)	0 (0)	0.54
Body pain	7 (15)	4 (12)	3 (20)	0.66
Underlying diseases: n (%)				
Hypertension	6 (13)	3 (9)	3 (20)	0.42
Diabetes mellitus	2 (4)	2 (6)	0 (0)	
Hyperthyroidism	1 (2)	1 (3)	0 (0)	
Baseline laboratory parameters Median [Q1 – Q3]				
CRP: mg/dL (n = 46)	47.5 [22.1 – 64]	46 [22 – 60]	53 [30.2 – 76]	0.29
D-Dimer: ng/ml (n = 40)	745.5 [423.1 – 1863]	1500 [700 – 2500]	693 [300 – 1043]	0.03
LDH: U/L (n = 45)	546[365 – 913]	529 [364 – 970]	617 [365 – 913]	0.69
WBC × 10^9: cells/L (n = 48)	4.93 [2.2 – 8.3]	5.2 [2.1 – 8.8]	4.7 [2.7 – 6]	0.81
Neutrophils × 10^9: cells/L (n = 37)	3.8 [2.0 – 6.9]	4.9 [1.9 – 7.6]	3.3 [2.0 – 3.8]	0.10
Lymphocytes × 10^9: cells/L (n = 38)	0.70 [0.44 – 1.51]	0.67 [0.36 – 1.51]	0.81 [0.53 – 1.87]	0.41
Platelets: (n = 48)	104.5 [34 – 168]	113 [37 – 180]	80 [14 – 144]	0.40
NLR (n = 37)	5.06 [2.35 – 9.51]	5.06 [2.35 – 16.96]	4.96 [1.77 – 5.66]	0.34
PLR (n = 38)	138.6 [54.0 – 224.0]	155.8 [65.8 – 295.8]	107.7 [8.9 – 178.7]	0.08
Hemoglobin: g/dL (n = 48)	9.5 [8.1 – 10.6]	9.8 [8.3 – 10.5]	8.3 [7.9 – 11.2]	0.46
Ferritin: pg/ml (n = 32)	1946 [761 – 4350]	1375 [624 – 3370]	3925 [1789 – 5180]	0.12

Fisher's exact test and Mann-Whitney U test were used to compare categorical and numeric variables, respectively. CRP: C-reactive protein; LDH: Lactate dehydrogenase; WBC: White blood cell; NLR: Neutrophil to lymphocyte ratio; PLR: Platelet to lymphocyte ratio; N: Number

medical symptoms, and medical drug usage history were gathered from the health information system (HIS) and health records. Routine blood examinations, including complete blood count, D-Dimer, serum ferritin, lactate dehydrogenase (LDH), and C-reactive protein (CRP), were also extracted at admission. Furthermore, the physician examined the data to check for any discrepancies. The follow-up time was calculated from admission to the day of discharge or death.

Statistical analysis

This study aimed to assess the effect of chemotherapy on death and ventilation in MM patients with COVID-19 infection. Log-binomial regression was used to assess this relationship; baseline white blood cells (WBC), CRP, and platelet counts were adjusted in multivariable analysis. Moreover, the relative risk (RR) and its corresponding 95% confidence interval (CI) were presented to illustrate the magnitude of the association. The *P*-values were reported as two-tailed, and $P \leq 0.05$ was considered statistically significant. All tests were performed using Stata version 14.

Ethics approval and consent to participate

The study was carried out in accordance with the Helsinki Declaration (IV Adaptation). The study protocol was approved by the Research Ethics Committees of the Vice-Chancellor in Research, Medical University of Isfahan (approval

Table 2. Association between receiving chemotherapy and ventilation or death

Model	Ventilation		Death	
	RR (95% CI)	*P*	RR (95% CI)	*P*
Model A[a]	1.21 (0.37 , 3.94)	0.75	1.02 (0.37 , 2.80)	0.97
Model B[b]	1.22 (0.37 , 3.96)	0.74	1.02 (0.38 , 2.76)	0.97
Model C[c]	1.17 (0.37 , 3.71)	0.79	1.00 (0.39 , 2.61)	0.99

Log-binomial regression was used to determine the relationship between chemotherapy and ventilation or death. [a]No adjustment; [b]adjustment for baseline WBC; [c]adjustment for baseline WBC and CRP; RR: Risk ratio; CI: Confidence interval; WBC: White blood cell; CRP: C-reactive protein

ID: IR.MUI.MED.REC.1400.213). In view of the retrospective nature of the study, the need for individual patient consent was waived by the research ethics committee as data protection safeguards were in place.

Results

Patients' characteristics

Our cohort comprised 48 patients, with a mean age of 66 years (standard deviation 12). 33 patients received Bortezomib within the last three months, while 15 did not. Of the total cohort, 30 patients were men, and 19 (58%) received Bortezomib. The median length of hospitalization in both groups was seven days (Table 1).

Association between receiving chemotherapy and ventilation or death

Table 2 shows that there was no significant relationship between the use of Bortezomib and ventilation (RR = 1.21; 95% CI: 0.37 to 3.94; $P = 0.75$). When adjusted for baseline WBC count, CRP, and platelet levels, the relationship became even weaker (RR = 1.18; 95% CI: 0.37 to 3.78; $P = 0.78$). Furthermore, Bortezomib use did not have a significant effect on mortality in either the crude or adjusted models (RR = 1.02; 95% CI: 0.37 to 2.80; $P = 0.97$; RR = 0.99; 95% CI: 0.38 to 2.58; $P = 0.99$, respectively).

Discussion

The results of our study showed that Bortezomib chemotherapy regimens did not affect the symptoms, severity, and mortality of patients. Previous studies have demonstrated that patients with malignancies, including MM, have a higher risk of mortality, developing severe forms of COVID-19, and requiring mechanical ventilation than the general population.[15] Cancer patients also experience shorter hospital stays, and their condition deteriorates rapidly,[16] which underscores the need for better and greater care by healthcare providers.

Our study revealed that the most common symptoms of MM patients infected with COVID-19 are fever, cough, and dyspnea. A meta-analysis by Zarifkar et al. also reported that the most common symptoms of cancer patients are non-specific, including fever and dyspnea.[3] The prevalence of non-specific symptoms like fever and dyspnea is not significantly different in cancer groups compared to the general population.[16] However, although symptoms like cough and fever have a high sensitivity for COVID-19, their specificity is low.[17] Thus, healthcare providers should not base their decision to rule in or rule out COVID-19 on symptoms alone, especially for suspected cancer patients. Our results also showed that receiving Bortezomib chemotherapy regimens did not affect the symptoms of MM patients.

The laboratory test results at admission also showed no significant differences between the groups, except for D-Dimer. D-Dimer is a biomarker that could have been affected by chemotherapy,[18] which explains why the difference between the groups was significant. Both groups had lymphopenia, which is a risk factor for severity and mortality in COVID-19. Having low lymphocytes in both groups indicates that all MM patients, regardless of whether they received Bortezomib or not, are at a higher risk of severity and mortality.[19]

Our study also demonstrated that the Bortezomib chemotherapy regimen did not affect the severity and mortality of patients. Previous studies suggest that laboratory tests such as WBC and CRP are predictors of severity and mortality in the general population and cancer patients. We

also adjusted the baseline WBC, CRP, and platelet[20,21] levels, but the results did not change, and there was no significant difference between the groups.

Most current studies have included all types of cancers or all hematological cancers together. Consequently, the proportion of each cancer type affected the results. Booth et al. and Cattaneo et al. have shown that active treatments are a risk factor for poor outcomes in patients with hematological malignancies.[22, 23] In contrast, Sanchez-Pina et al. concluded that the decisions for systemic anticancer treatment modifications or delays are case-by-case decisions.[24] A report from a tertiary center on MM patients also concluded that cancer treatments like chemotherapy did not affect mortality.[25] However, it is noteworthy that the sample size of this study was small, with only 58 patients.

Limitations

This study has limitations that should be considered. The first and major limitation of this study is the small sample size. Therefore, it is strongly recommended to conduct multi-institutional studies to create better evidence. Another limitation of the study is the short follow-up time. We only followed patients during their hospital admission. Studies with longer follow-up periods can help to determine the long-term effects of Bortezomib on the survival of patients with multiple myeloma. As many countries, including Iran, have vaccinated their communities, including cancer patients, it is recommended to conduct studies on vaccinated patients, as vaccination is an important variable that can affect the results.

Conclusion

Determining whether to continue chemotherapy in cancer patients infected with COVID-19 or not is a major concern for oncologists. Our study showed that receiving Bortezomib chemotherapy regimens did not worsen the symptoms and prognosis of multiple myeloma patients infected with COVID-19. However, more studies with larger sample sizes and longer follow-up periods are needed to create better evidence on this subject.

Availability of Data and Materials

The datasets of the study are not publicly available due to patient confidentiality, but a de-identified version will be made available from the corresponding author on reasonable request.

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Conflict of Interest

None delared.

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Evaluation of *TRAF3IP2* Gene Expression in Brain Tumor Tissue of Patients with Glioblastoma Multiforme in Comparison to Non-Tumoral Brain Tissue

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Abstract

Background: Glioblastoma, not otherwise specified (NOS), is the most common primary malignant brain tumor. The *TRAF3IP2* gene is an upstream regulator responsible for activating multiple proinflammatory pathways that could influence tumor size, angiogenesis, aggressiveness, and metastasis. In the present study, we aimed to investigate and assess the *TRAF3IP2* gene expression in brain tumor tissue of patients with glioblastoma, NOS and compare it with non-neoplastic brain tissue.

Method: In this case-control study, biopsies were obtained from 15 surgically glioblastoma, NOS removed block samples and 15 non-neoplastic brain tissue samples containing normal white and gray matter as controls. Ribonucleic acid (RNA) was isolated and reverse-transcribed to complementary DNA (cDNA). Quantitative polymerase chain reaction (qPCR) was then carried out to measure *TRAF3IP2* gene expression.

Results: We evaluated data from 30 cases, divided into two groups: case (N = 15) and control (N = 15). Based on our data, the expression of the *TRAF3IP2* gene was 6.95 ± 0.65 times higher in glioblastoma multiforme tissue compared with controls ($P < 0.05$). We also found no significant difference in *TRAF3IP2* gene expression between genders ($P = 0.452$), and there was no significant correlation between *TRAF3IP2* gene expression and age ($P = 0.745$).

Conclusion: The expression of the *TRAF3IP2* gene was almost seven times higher in glioblastoma, NOS brain tissue compared with normal brain samples. This finding could have significant clinical and therapeutic implications.

Keywords: Glioblastoma multiforme, Gene expression, Case-control study

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Introduction

Glioblastoma, not otherwise specified (NOS), is one of the most invasive astrocytic tumors.

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Glioblastoma is the most common primary malignant tumor of the central nervous system (CNS) that occurs in the spinal cord or brain.[1, 2] The overall prevalence of this cancer is 2 to 3 per 100,000 people.[3, 4] The most common site of glioblastoma tumors is the supratentorial region. Glioblastoma, NOS accounts for 20% of all intracranial tumors and 60% of astrocytic tumors, and is more common in men over 60 years of age.[5, 6]

Symptoms of the tumor depend on its location and include headache, nausea, seizures, cranial nerve disorders, blurred vision, irritability, increased intracranial pressure, neutrophilic leukocytosis, decreased level of consciousness, and more. Complications of glioblastoma, NOS tumors depend on the site of the tumor growth.[3, 7]

The primary treatment strategy for glioblastoma, NOS is surgery; however, the prognosis of the tumor is poor, even when the most invasive treatment is used, which includes radiation therapy, chemotherapy, and surgery.[8] Standard treatment for glioblastoma, NOS includes maximal surgical resection of the tumor followed by radiotherapy between 2 and 4 weeks after surgery to remove tumor debris.[9, 10]

So far, the only known risk factors are genetic syndromes and radiation.[11] In addition to malignant cells, glioblastoma lesions include non-malignant cells, including inflammatory cells, endothelial cells, neural cells, and glial cells. Malignant and non-malignant cells secrete pro-inflammatory mediators that cause tumor growth, metastasis, and invasion.[12] Many of these mediators are secreted in response to genes such as NF-κB. As a result, NF-κB activity causes further tumor growth and a poor prognosis.[13]

TRAF3IP2 is the gene responsible for an upstream regulator for NF-κB activity, and as a result, its expression activates multiple pro-inflammatory pathways.[14, 15] NF-κB is a protein complex that controls DNA transcription, cytokine production, and cell survival. NF-κB is crucial in regulating the immune response to infection.[15] Some studies have also targeted the expression of pro-angiogenic mediators, including VEGF, by targeting *TRAF3IP2*. By silencing *TRAF3IP2* expression, NF-κB activity can be inhibited, thus reducing tumor growth, metastasis, and angiogenesis.[16] These studies highlight the possible therapeutic roles of *TRAF3IP2* gene suppression in tumors. Previous studies have indicated the potential of the *TRAF3IP2* gene in glioblastoma, NOS treatments, but there is still

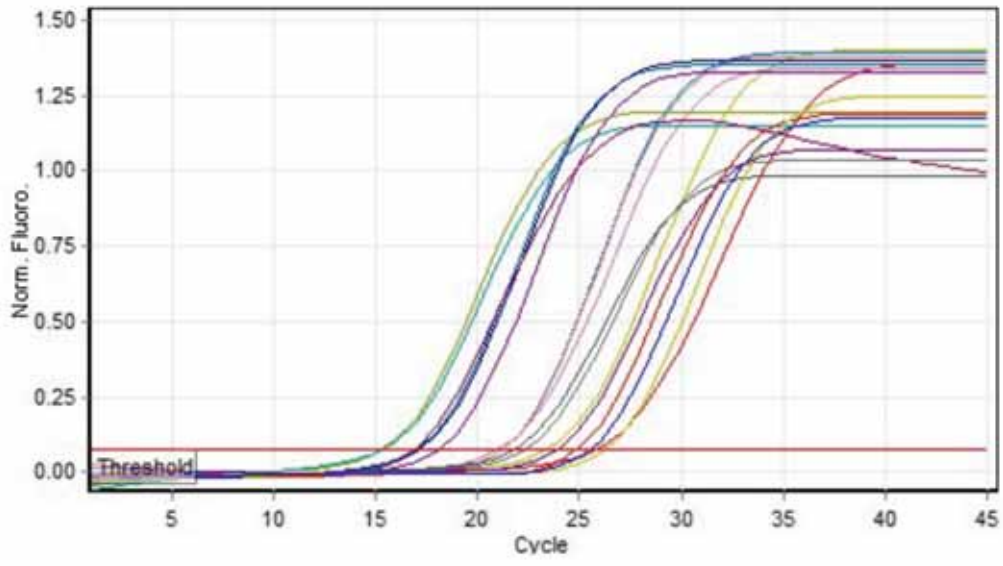

Figure 1. This figure displays the amplification graph of real-time PCR for *TRAF3IP2*. The x-axis represents the cycle number, the y-axis shows the change in fluorescent intensity, and the red horizontal line denotes the threshold.
Norm.Fluoro: Normal Fluorosecence; PCR: Polymerase chain reaction

much to discover in this regard, including the amount of gene expression in glioblastoma, NOS tissue.[17]

In this study, we aim to investigate and assess the *TRAF3IP2* gene expression in brain tumor tissue of patients with glioblastoma, NOS, considering the limited studies on this issue and the importance of glioblastoma, NOS and the potential properties of *TRAF3IP2* expression in glioblastoma, NOS tissue.

Materials and Methods

Ethics statement

The study protocol was approved by the Research Committee of Isfahan University of Medical Sciences and confirmed by the affiliated Ethics Committee (ethics code: IR.MUI.MED.REC.1398.481). Tumor specimens were obtained from the pathology banks of patients diagnosed with glioblastoma, NOS at Al-Zahra University Hospital in Isfahan. All data related to human material used in this case-control study were managed using anonymous numerical codes.

Clinical samples and histology

The inclusion criteria for the study were accessibility of the pathology samples, availability of samples in the pathology unit of Al-Zahra hospital, and a definitive diagnosis of glioblastoma, NOS. The exclusion criterion was damaged samples. The samples were selected from the patient lists in the Treasury. Based on these criteria, 15 cases of glioblastoma, NOS were selected for gene expression analysis. All specimens were primary glioblastoma, NOS, and the patients had not undergone neoadjuvant therapy prior to surgery. Expert pathologists made the diagnosis of glioblastoma, NOS, according to the 2021 WHO criteria.[18] In addition to the glioblastoma, NOS samples, 15 non-neoplastic brain biopsy samples containing normal white and gray matter tissues were obtained from the pathology bank. These control brain specimens were collected during the biopsy of patients with hemorrhagic brain injuries, brain arteriovenous malformations (AVMs), or colloid cysts in the brain. During the biopsy, we evaluated the normal brain tissues. The samples included 21 males and 9 females with a mean age of 49.26 ± 13.91 years.

All biopsy samples were placed into a 10% neutral buffered formalin solution, fixed at room temperature, and then processed routinely into a paraffin-embedded tissue block (FFPE).

Preparation of FFPE tissue sections and RNA extraction

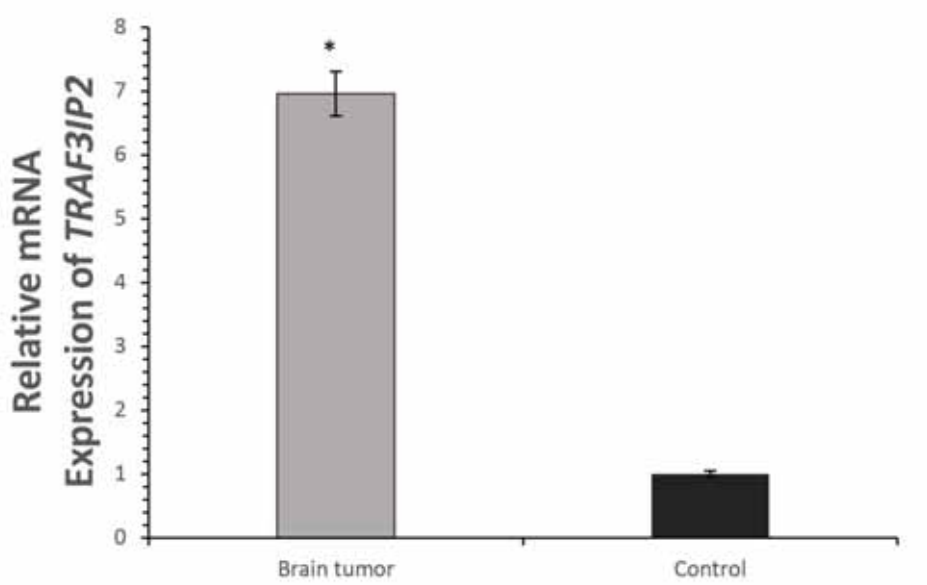

Figure 2. This figure illustrates the relative comparison of *TRAF3IP2* gene expression among samples (*$P < 0.05$). The relative expression of *TRAF3IP2* was almost seven times higher in brain tumors than in normal tissues.

To extract RNA more efficiently, xylene was used to remove the paraffin cuts before the extraction process, followed by ethanol-based rehydration. The manually dissected 50 mg samples were placed into 1.5 mL RNase-free microtubes, and 1 mL xylene was added to each sample, which was then homogenized using vortexing. Subsequently, the microtubes were centrifuged for 5 minutes at 1400 rpm in the Beckman Coulter Microfuge 22r, the xylene was discarded, and the pellet button was rinsed twice with absolute ethanol (Merk KGaA, CAS-NO: 64-17-5).

After preparing the samples, RNA was extracted from selected blocks by manual TRIzol™ method (Yekta Tajhiz Co., Iran, cat. No.: YT9064), according to the protocol reported by Jordon-Thaden and colleagues in 2015.[19] The tissue was digested by incubating the samples in 180 µL TE buffer (1x TE buffer, Sinacolon, Cat. Na EP5071) and 20 mg/mL proteinase K (Adbio, Ref. 91321). First, the sample was incubated at 56°C for two hours, and then, proteinase K was inactivated by raising the temperature to 90°C for one hour. Finally, samples were subjected to an RNA extraction process according to the Trizol method previously described by Jordon-Thaden and colleagues in 2015.[19] All RNA samples were stored frozen at -80°C until used.

Quantitative real-time polymerase chain reaction (qPCR)

The reverse transcription (RT) reaction was carried out using reverse transcriptase enzyme and random hexamer primers, according to the manufacturer's instructions (Addbio kits, Korea, Ct. No.: 22701).

Real-time qPCR was set up according to an Opticon Instrument (Bio-Rad Laboratories, Hercules, CA) using SYBR Green reagent for the detection of PCR products. PCR reactions were performed using 2 µL cDNA with 10 µL of a SYBR Green master mix (GeNet Bio-Korea, Ct. No.: 70201) containing forward and reverse primers, MgCl2, and SYBR Green. Primers for the *TRAF3IP2* gene were designed using Oligo 7 software. The *TRAF3IP2* primers were as follows: forward, 5'-GCTTTATTCAGACTTACACCGAT and reverse, 5'-AGTCTAATAC TTTGATTGTAGCCA. The specificity of the primers was checked in the nucleotide database (NCBI, nucleotide BLAST). Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) served as a housekeeping gene, and the expression level of target genes was normalized to *GAPDH*.

Table 1. *TRAF3IP2* gene expression, gender, and age

		***TRAF3IP2* gene expression**	
		Case	**Control**
Sex	Male	6.98 ± 0.62	1 ± 0
	Female	6.56 ± 1.28	1 ± 0
	P value1	0.452	-
Age	Pearson correlation	0.1	-
	P value 2	0.745	-

Statistical analysis

The obtained data were entered into the Statistical Package for Social Sciences (SPSS) version 24. Quantitative data were reported as mean ± standard deviation, and qualitative data were reported as frequency distribution (percentage). Independent t-test and chi-square were used to analyze the data. Relative quantification of gene expression was calculated based on the log $2^{-\Delta\Delta Ct}$ formula.[19, 20] A *P*-value < 0.05 was considered the significance threshold.

Results

In the present study, data from 30 cases were evaluated and divided into two groups: case (N = 15) and control (N = 15). The study population consisted of 21 males (70%) and 9 females (30%) with a mean age of 49.26 ± 13.91. There were no significant differences between the two groups regarding gender ($P = 0.378$) and age ($P = 0.70$). From the amplification curve, it can be seen that the quality of the real-time qPCR is fine (Figure 1).

Based on our data, the expression of the *TRAF3IP2* gene was relatively 6.95 ± 0.65 times higher compared with controls ($P < 0.05$) (Figure 2). These data suggest significantly higher *TRAF3IP2* gene expression in glioblastoma, NOS samples compared with normal brain tissue, which could, in turn, be used in pathological practice.

Further analysis found no significant difference regarding *TRAF3IP2* gene expression between genders ($P = 0.452$), and there was no significant

correlation between *TRAF3IP2* gene expression and age ($P = 0.745$) (Table 1).

Discussion

We evaluated the expression of the *TRAF3IP2* gene in glioblastoma, NOS brain samples and found that its expression was almost seven times higher compared with that of normal brain tissue. This finding highlights the importance of the *TRAF3IP2* gene in the pathogenesis of glioblastoma, NOS. Additionally, our study revealed no significant differences in *TRAF3IP2* gene expression based on age or gender.

Previous studies have also evaluated *TRAF3IP2* gene expression in glioblastoma, NOS. In 2018, Alt et al. assessed *TRAF3IP2* as a therapeutic target in glioblastoma, NOS. They demonstrated that the expression of *TRAF3IP2* was six times higher in glioblastoma, NOS tissue in both animal and human models. They investigated the therapeutic options using this gene and reported that silencing *TRAF3IP2* significantly inhibited the sphere-forming potential of malignant glioblastoma cells. They found that this silencing also suppressed activation of NF-κB and induction of proinflammatory mediators. Another important finding was that targeting *TRAF3IP2* suppressed the expression of various pro-angiogenic mediators.[17] The results of our study were consistent with these findings. However, we only evaluated the expression of the *TRAF3IP2* gene and did not assess possible therapeutic options.

In 2015, Silver et al. reported that gliomas and other primary brain tumors contain a subpopulation of cells that express stem cell-like properties (cancer stem cells or CSCs) and contribute to tumor growth and drug resistance, and possibly tumor recurrence. They suggested that the diagnosis of these genes could result in significant therapeutic progress.[21]

Several other studies have also assessed the expression of the *TRAF3IP2* gene in glioblastoma, NOS. However, the number of such studies is limited. Zan et al. conducted a bioinformatic study in 2019 in China. This study assessed an online microarray dataset, including 26 glioblastoma, NOS samples and six samples from normal brain tissue. The study showed that EPB41L4A-AS1, ZRANB2-AS2, XIST, HOTAIR, *TRAF3IP2*, TPT1-AS1, PVT1, and DLG1-AS1 genes play pivotal roles in the pathogenesis of glioblastoma, NOS. Based on this study, the expression of *TRAF3IP2* was significantly higher in glioblastoma, NOS samples.[22]

Shao et al. also showed the higher expression of *TRAF3IP2* in glioblastoma, NOS in a bioinformatics study conducted in 2018. This study also highlighted the possibility of lncRNAs being utilized in glioblastoma, NOS.[23] Our survey findings were consistent with these studies showing a higher expression of the *TRAF3IP2* gene in glioblastoma, NOS.

The *TRAF3IP2* gene expression has been evaluated in breast cancer, and in 2020, Alt and colleagues investigated the roles of Rab27a, a player in exosome release, and *TRAF3IP2*, an inflammatory mediator, in the development and metastasis of breast cancer. This study also demonstrated that *TRAF3IP2* has higher expression in metastatic breast cancer and showed that silencing this gene blocked the interaction between tumor cells and mesenchymal stem cells injected into the contralateral gland. As a result, *TRAF3IP2* gene suppression could play an essential role in breast cancer.[24] These data suggest that *TRAF3IP2* could be a novel therapeutic option for cancer prevention and treatment.

The importance of the *TRAF3IP2* gene has been mentioned in different previous studies, and some have suggested using this gene in cancer treatment. Based on a report by Alt and colleagues in 2018, the *TRAF3IP2* gene could be a novel therapeutic target in glioblastoma, NOS growth, and dissemination. This study highlighted the roles of *TRAF3IP2* gene suppression in reducing pro-inflammatory/pro-tumorigenic/pro-angiogenic mediators and kinesins.[17] In addition, increased expression of *TRAF3IP2* was established in glioblastoma, NOS brain xenograft models. This study also revealed that targeting *TRAF3IP2* suppresses angiogenesis by decreasing the secretion of several pro-angiogenic mediators, including VEGF, resulting in decreased angiogenesis.[25] In this study, we showed

significantly higher expression of the *TRAF3IP2* gene in glioblastoma, NOS tissue, which was similar to their findings. We believe effective therapeutic strategies could be developed by targeting the *TRAF3IP2* gene.

Furthermore, some studies have also evaluated *TRAF3IP2* gene expression in other tumors and ischemic conditions. In recent research by Alt et al. in 2020, they described that the *TRAF3IP2* gene has significant roles in breast cancer growth and metastasis and contributes to inflammatory situations. A crucial finding of their study was that by targeting and suppressing the expression of the *TRAF3IP2* gene, it suppresses tumor growth as well as macro- and micro-metastasis by reducing LTα (Lymphotoxin Alpha) and PDGFA (Platelet Derived Growth Factor Subunit A) expression in MDA-MB231 cells.[24] Another study by Erikson and colleagues explained that the *TRAF3IP2* gene plays a causal role in myocardial ischemia/reperfusion injury, dysfunction, and adverse remodeling. In this regard, suppressing this gene significantly inhibited myocardial injury and adverse remodeling.[26] These data support the potential of *TRAF3IP2* gene suppression in different conditions.

To the best of our knowledge, all the studies conducted in the common field with the present study were based on bioinformatics and in vitro studies using glioblastoma cell lines and animal models. Despite these valuable studies, there is a lack of investigation of *TRAF3IP2* expression levels in human tumor samples. Notably, this study showed that the expression levels of *TRAF3IP2* in human samples are even higher than those obtained in previous studies. According to our results and these previous reports, *TRAF3IP2* gene targeting could be a novel therapeutic strategy in glioblastoma, NOS, and other conditions, primarily due to its higher expression rates in glioblastoma, NOS tissue.

The limitations of our study included restrictions in the study samples due to the challenging sampling process and the evaluation of only the expression of the *TRAF3IP2* gene. We recommend that further studies concentrate on the effects of suppression and silencing of this gene in cancer therapy based on its potential as a therapeutic target.

Conclusion

The expression of the *TRAF3IP2* gene was almost seven times higher in brain tissue from glioblastoma, NOS, compared with normal brain samples. This finding could have high clinical and therapeutic importance, and we believe that further studies should be conducted in this regard.

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Conflict of Interest

None declared.

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Original Article
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Second-line Modified GTX versus Gemcitabine-Nab-Paclitaxel (GmAb) Following First-Line FOLFIRINOX in Advanced Pancreatic Cancer: A Retrospective Analysis at the American University of Beirut Medical Center (AUBMC)

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Abstract

Background: Pancreatic cancer is characterized by its generally poor prognosis and ranks seventh worldwide in cancer-related mortality. We previously conducted a prospective study on the use of modified GTX regimen (a combination of gemcitabine, docetaxel, and capecitabine), which has appreciable activity and is well-tolerated, in this setting. We compared the efficacy of GTX regimen versus Gemcitabine-nab-paclitaxel (GmAb) as second-line chemotherapy in advanced pancreatic cancer patients receiving first-line therapy with FOLFIRINOX.

Method: This retrospective chart review aimed to collect and record data corresponding to patients diagnosed with advanced pancreatic cancer at the American University of Beirut Medical Center who received FOLFIRINOX as first-line chemotherapy and who then had GTX or GmAb as second-line treatment between 2013 and 2019. We measured the progression-free survival, overall survival, and toxicity of GTX versus GmAb as second-line treatment for pancreatic adenocarcinoma at AUBMC.

Results: The median overall survival for the GmAb group was around 52 months, which is greater than that of the GTX group, which was 25 months. 26.7% of patients who received GTX required dose reduction starting from cycle one, while only 3.1% of those who received GmAb required dose reduction from cycle one. 38.7% of patients who received GmAb did not have anemia throughout the course of treatment, while the majority of patients who received GTX, 93.3%, had grade I anemia.

Conclusion: Our data show that GmAb is a possibly better second-line treatment option than GTX with better tolerance to the dose, less anemia, and a better survival profile. More studies are needed with a larger sample size and a prospective design to prove such a possible difference between the two regimens.

Keywords: Gemcitabine, FOLFIRINOX, Pancreatic neoplasms, Second-line chemotherapy

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Introduction

Pancreatic cancer is characterized by a generally poor prognosis and ranks seventh worldwide in cancer-related mortality. It accounts for about 3% of all cancers in the US and Europe and about 7% of all cancer deaths.[1, 2] While surgical resection represents the best curative management approach, only 10% of patients are resectable at diagnosis, and the remaining either have metastatic disease (50%) or locally advanced disease (30%).[1-3] Despite surgical resection, the 5-year overall survival (OS) remains limited to around 20%, and 30% of patients tend to develop early recurrence, with the majority eventually relapsing.[2] Moreover, induction chemotherapy followed by radiation therapy is the recommended first-line approach for locally advanced unresectable disease. The preferred regimens for pancreatic cancer remain FOLFIRINOX or gemcitabine and nab-paclitaxel.[4] However, there are almost no prospective studies regarding second-line regimens. As such, there is no consensus regarding a standard approach in the second-line setting for advanced pancreatic cancer.

Specifically, there is a paucity of studies that explore the possible regimens following the use of fluoropyrimidine-based regimens. This is of particular importance with the increasing use of FOLFIRINOX (5-fluorouracil, leucovorin, irinotecan, and oxaliplatin) in the first-line setting. There is considerable interest in the choice of second-line chemotherapy, especially gemcitabine-containing regimens. We have previously conducted a prospective study on the use of the modified GTX regimen (a combination of gemcitabine, docetaxel, and capecitabine), which has appreciable activity and is well-tolerated in this setting.[5,6]

Here, we compared the efficacy of the GTX regimen versus gemcitabine-nab-paclitaxel (GmAb) as second-line chemotherapy in advanced pancreatic cancer patients receiving first-line therapy with FOLFIRINOX.

Aim and hypothesis

Our aim was to compare the use of the GTX regimen and GmAb as second-line chemotherapy in patients with advanced pancreatic cancer. We hypothesized that comparing the use of GTX or GmAb as second-line chemotherapy in advanced pancreatic cancer patients could guide treatment choice in this setting.

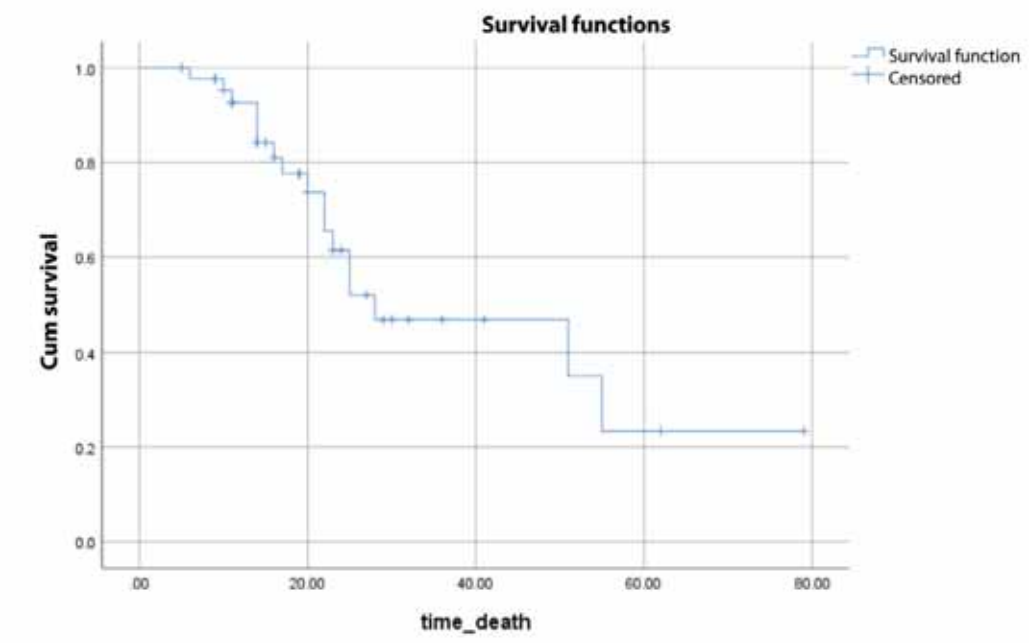

Figure 1. Kaplan-Meier survival curve for the whole patient population (GTX and GmAb)
Cum: Cumulative; GTX: Combination of gemcitabine, docetaxel and capecitabine; GmAb: Combination of gemcitabine and nab-paclitaxel

Materials and Methods

This is a retrospective chart review that aimed to collect and record data from patients diagnosed with advanced pancreatic cancer at the American University of Beirut Medical Center who received FOLFIRINOX as first-line chemotherapy and then had GTX or GmAb as second-line treatment between 2013 and 2019. Informed consent was obtained from the participants, and data collected and recorded included patient demographics (age, gender, nationality), descriptive characteristics (past medical and surgical history, risk factors), disease characteristics (staging, grading), imaging findings, treatment plans, tumor responses to treatment, and adverse events. The data cut-off was at June 15, 2021. The ethics approval to review the charts was obtained from the Institutional Review Board at the American University of Beirut (IRB ID: BIO-2019-0092). Data was collected from the patients' Paper Medical Charts and Electronic Health Records corresponding to eligible patients. They were recorded on Data Collection Sheets and stored in a locked cabinet. We measured the progression-free survival (PFS), OS, and toxicity of GTX versus GmAb as second-line treatment for pancreatic adenocarcinoma at AUBMC. Inclusion criteria were patients aged 18 years or older with an ECOG performance status of 0, 1, or 2, with histologically proven pancreatic adenocarcinoma, metastatic or locally advanced unresectable disease, who progressed on first-line FOLFIRINOX and received GTX or GmAb as second-line treatment. Exclusion criteria were having an ECOG performance status greater than 2 and second-line therapy not involving GTX or GmAb. All patients who progressed on first-line FOLFIRINOX were recruited by their primary oncologists to receive second-line chemotherapy when they had a performance status of 0, 1, or 2 and agreed to continue treatment. There was no pre-set number of planned chemotherapy cycles in the patient population, as the treatment in their second-line course was planned until progression of disease and as long as tolerated. Outcome was assessed by imaging every 6 months. An increase in the size of the primary and/or metastatic tumors, and/or the development of new regional or

Table 1. Patients demographics and baseline characteristics

Gender	
Male	31 (60.8%)
Female	20 (39.2)
Nationality	
Lebanese	40 (78.43)
Other	11 (21.57)
Smoker	
Never	27 (52.94)
Yes	24 (47.06)
Alcohol drinker	
No	42 (82.35)
Yes	9 (17.65)
Diabetes	
No	24 (47.06)
Yes	27 (52.94)
Hypertension	
No	40 (29.40)
Yes	11 (21.60)
Comorbidities	
More than one (kidney disease, liver disease, cardiac disease, thyroid disease, dyslipidemia, and hypertension)	12 (23.53)
One	39 (76.47)
Pancreatic tumor location	
Uncinate/head	20 (39.21)
Tail	13 (25.49)
Neck/Body	13 (25.49)
Body/Tail	5 (9.80)
Pancreatic tumor size	
3 cm	23 (45.10)
2 cm	15 (29.41)
1, 4, or 5 cm	13 (25.49)
Lymph node involvement	
None	21 (41.18)
One	16 (31.37)
Two	14 (27.45)
Distant metastasis	
No	21 (41.18)
Liver	17 (33.33)
Lungs	4 (7.84)
Peritoneum	1 (1.96)
More than one site	8 (15.69)
Stage	
IB	5 (9.80)
IIA	4 (7.84)
IIB	4 (7.84)
III	8 (15.69)
IV	30 (58.82)
Surgery	
Whipple	6 (11.76)
Distal pancreatectomy	1 (1.96)
None	44 (86.27)

Table 2. Distribution of "dose reduction" among the two arms

	Dose reduction ($P = 0.011$)			Total
	At cycle one inclusive	At subsequent cycles (after cycle one)	No dose reduction	
GTX	4 (26.7%)	0 (0.0%)	11 (73.3%)	15 (100%)
Gemcitabine nab-paclitaxel	1 (3.1%)	7 (21.9%)	24 (75.0%)	32 (100%)
Total	5	7	35	47

GTX: Combination of gemcitabine, docetaxel and capecitabine

distant metastasis was defined as progression. PFS was calculated at the patient level as the interval between the initiation of second-line treatment and disease progression. Progression of disease was defined as per RECIST guidelines. OS was calculated as the time from the second-line treatment until death from any cause or the last follow-up. Toxicity was evaluated by the Common Terminology Criteria for Adverse Events, version 4.0 (CTCAE v4.0). All analyses were performed using SPSS statistical software (Chicago, IL, USA).

Results

Baseline characteristics

Table 1 presents the demographics and baseline characteristics of the patients. The majority of patients (88.2%) had pancreatic adenocarcinoma, while 11.8% had pancreatic adenocarcinoma with mucinous features. Of the patients, 20 (39.21%) had their tumor located at the uncinate/head of the pancreas, 13 (25.49%) had it at the pancreatic tail, 13 (25.49%) had it at the pancreatic neck/body, while 5 (9.80%) had their tumor located at the pancreatic body/tail. The tumor size was approximately 3 cm in 23 (45.1%) patients and 2 cm in 15 (29.4%) patients, while the remaining patients had a tumor of size 1, 4, or 5 cm. 21 patients (41.18%) had no lymph node involvement, 16 (31.37%) had one lymph node involvement, and 14 (27.45%) had 2 lymph node involvements. The majority of patients (58.8%) had metastasis, where 17 (33.33%) had metastasis to the liver, 4 (7.84%) had metastasis to the lungs, 1 (1.96%) had metastatic disease to the

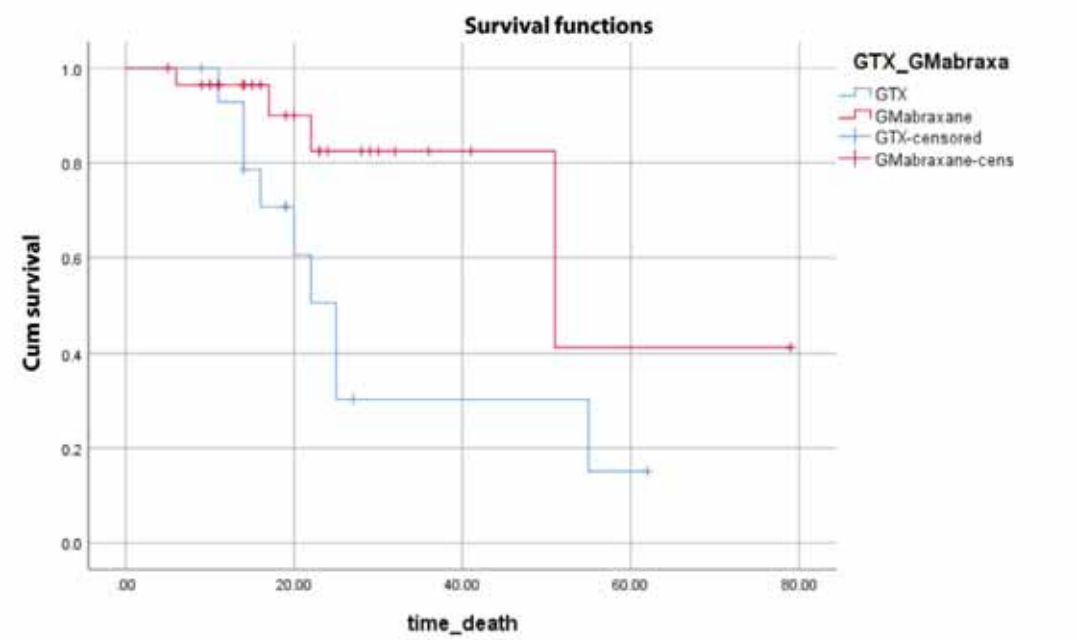

Figure 2. Kaplan-Meier survival curve for each of the two arms (GTX and GmAb)
Cum: Cumulative; GTX: Combination of gemcitabine, docetaxel and capecitabine; GmAb: Combination of gemcitabine and nab-paclitaxel

Table 3. Distribution of "anemia" among the two arms

	Anemia ($P = 0.047$)			Total
	No anemia	CTCAE Grade I	CTCAE grade I	
GTX	1 (6.7%)	14 (93.3%)	0 (0.0%)	15 (100%)
Gemcitabine nab-paclitaxel	12 (38.7%)	18 (58.1%)	1 (3.2%)	31 (100%)
Total	13	32	1	46

GTX: Combination of gemcitabine, docetaxel and capecitabine; CTCAE: Common Terminology Criteria for Adverse Events

peritoneum, and 8 (15.69%) had metastasis to more than one site. The majority of patients (58.82%) had stage IV at diagnosis. Six patients (11.76%) underwent the Whipple procedure, and 1 (1.96%) had a distal pancreatectomy. 32 patients (62.7%) did not receive radiation therapy, while 16 (31.4%) received radiation therapy.

The majority of patients (96.1%) received as the first-line regimen, 5-Fluorouracil, Irinotecan, and Oxaliplatin. Only 1 patient (2.0%) stopped this regimen due to neutropenia and mucositis, while the remaining patients stopped this regimen due to the progression of the disease. On progression, 39 patients (76.5%) had new distant metastasis; 22 of which (43.1%) had metastasis to the liver, while 6 (11.8%) had distant metastasis to the lungs.

Disease course in the whole patient population

15 patients (29.4%) received, GTX as a second-line regimen, while 34 (66.7%) received GmAb as a second-line therapy. The majority of patients (76.5%) did not require a dose reduction, while 5 (9.8%) had a dose reduction in cycle 1, and 7 (13.7%) had a dose reduction in subsequent cycles. 12 (23.5%) had their regimen changed due to the progression of the disease. 11 (21.6%) had stable disease after the second regimen, while 34 (66.7%) had disease progression. At the time of data review, 28 (54.9%) were alive, while 20 (39.2%) had died. Figure 1 shows the Kaplan-Meier survival curve for the entire patient population. The median OS for the whole patient population was approximately 24 months.

The majority of patients (70.6%) had anemia, while only 10 (19.6%) had neutropenia, and 14 (27.5%) had thrombocytopenia during their treatment. Only three patients (5.9%) had mucositis and oral thrush, 5 (9.8%) had nausea and vomiting, 3 (5.9%) had diarrhea, and 6 (11.8%) had fatigue. 45 (88.2%) did not have infections during their treatment course. Only two patients (3.9%) had liver toxicity, and one (2.0%) had neurological toxicity. Other adverse events included muscle spasm reported in one patient (2.0%), and abdominal pain and decreased appetite in 1 patient (2.0%).

The two arms

The variables and outcomes that showed statistically significant differences between the two arms were dose reduction, anemia, and death (Tables 2-4). The majority of patients who received either the GTX or GmAb regimens did not require dose reduction, with 73.3% and 75.0% of patients from each arm, respectively, not requiring dose modification. However, 26.7% of patients who received GTX required dose reduction starting from cycle one, while only 3.1% of those who received GmAb required dose reduction from cycle one. Instead, 21.9% of patients in the latter arm required dose reduction at subsequent cycles during the treatment course (Table 2, $P = 0.011$).

Of the patients who received GmAb, 38.7% did not have anemia throughout the course of treatment, 58.1% had CTCAE grade I anemia, and 3.2% had grade II anemia. On the other hand, the majority of patients who received GTX, 93.3%, had grade I anemia (Table 3, $P = 0.047$). 60% of patients who received GTX had died at the data cut-off time, while 84.4% of patients who received GmAb were still alive (Table 4, $P = 0.005$). The median follow-up was 24 months. Figure 2 shows the Kaplan-Meier survival curve for each of the two arms. The median OS for the

Table 4. Distribution of "death" among the two arms

	Death ($P = 0.005$)		Total
	Death	**No death**	
GTX	9 (60.0)	6 (40.0%)	15 (100%)
Gemcitabine nab-paclitaxel	5 (15.6%)	27 (84.4%)	32 (100%)
Total	14	33	47

GTX: Combination of gemcitabine, docetaxel and capecitabine

GmAb group was around 52 months, which was greater than that of the GTX group, which was 25 months ($P = 0.029$). Other variables did not show a statistically significant difference and are shown in table 5.

Discussion

There is currently no consensus regarding a standard approach in the second-line setting for advanced pancreatic cancer. This becomes particularly important with the increasing use of FOLFIRINOX in the first-line setting. There is considerable interest in the choice of second-line chemotherapy, especially gemcitabine-containing regimens. In this study, GmAb seemed to be a better second-line treatment option than GTX with better tolerance to the dose, less anemia, and a better survival profile. At the time of data review, 28 patients (54.9%) were alive, while 20 (39.2%) had died. The median OS for the GmAb group was around 52 months, which is greater than that of the GTX group, which was 25 months. 26.7% of patients who received GTX required a dose reduction starting from cycle one, while only 3.1% of those who received GmAb required a dose reduction from cycle one. 38.7% of patients who received GmAb did not have anemia throughout the course of treatment, while the majority of patients who received GTX, 93.3%, had grade I anemia.

Our data on the second-line regimen in advanced pancreatic cancer are particularly important since randomized trials provide little evidence of greater benefit from second-line therapy compared with best supportive care alone and because there is no clear consensus regarding the best second-line treatment option.[7] There has been an increased use of second-line chemotherapy particularly over the past decade. The use of second-line regimens is mainly for patients who maintain a good performance status. There is a limited number of randomized clinical trials that evaluated the role of second-line chemotherapy in metastatic pancreatic cancer. The first German CONKO trial showed that the combination of oxaliplatin, Leucovorin (LV), and 5-FU (OFF) resulted in better OS (4.8 months) compared with best supportive care (2.3 months).[8] This trial was discontinued, however, due to low patient accrual. Moreover, Nal-IRI is a liposomal encapsulated form of irinotecan, which improves the therapeutic index and prolongs its half-life. The NAPOLI-1 trial, a study of MM-398 with or without 5-FU/LV, versus 5-FU/LV in patients with metastatic pancreatic cancer, was a phase III clinical trial that included patients with metastatic pancreatic cancer and good performance status following treatment with gemcitabine.[9] The median OS benefit was 6.1 months for the combination of nal-IRI and 5-FU/LV compared with 4.2 months for the control arm of 5-FU/LV.[9] These above-mentioned trials; however, did not address second-line chemotherapy following first-line treatment with FOLFIRINOX, and the majority focused on the role of oxaliplatin in the second-line setting. This is what makes our study of particular relevance, where we focused on second-line treatment regimens not involving oxaliplatin, specifically GTX and GmAb following first-line treatment with FOLFIRINOX. Our study introduces GmAb as a possibly better second-line treatment option than GTX, with better tolerance to the dose, less anemia, and a better survival profile.

Our patient population reported a median OS of 24 months. Despite advancements in

Table 5. Distribution of other variables among the two arms

	GTX	Gemcitabine nab-paclitaxel	Total	*P*-value
Gender				0.542
Male	10	18	28	
Female	5	14	19	
Smoking status				0.758
Non-smoker	8	19	27	
Smoker	7	13	20	
Alcohol drinking				0.404
No alcohol	14	26	40	
Drinks alcohol	1	6	7	
Diabetes				0.758
No diabetes	7	13	20	
Has diabetes	8	19	27	
Tumor site				0.472
Uncinate/Head	5	14	19	
Body	3	6	9	
Tail	3	9	12	
Body and tail	4	3	7	
Distant metastasis				0.542
No metastasis	5	14	19	
Has distant metastasis	10	18	28	
Stage at diagnosis				0.680
Stage I	1	4	5	
Stage II	3	5	8	
Stage III	1	7	8	
Stage IV	10	15	25	
Radiation therapy				0.062
Did not receive radiation	13	17	30	
Received radiation	2	14	16	
Thrombocytopenia				0.748
No thrombocytopenia	11	21	32	
Had thrombocytopenia	4	10	14	
Mucositis/oral thrush				0.690
None	13	29	42	
CTCAE grade I	2	1	3	
CTCAE grade III	0	1	1	
Nausea/vomiting				1.00
None	13	28	41	
CTCAE grade I	2	3	5	
Diarrhea				1.00
None	14	29	43	
CTCAE grade I	1	2	3	
Infections				0.656
None	14	27	41	
Had infections	1	4	5	
Fatigue				0.651
No fatigue	13	27	40	
CTCAE grade I	1	5	6	

GTX: Combination of gemcitabine, docetaxel and capecitabine; CTCAE: Common Terminology Criteria for Adverse Events

pancreatic cancer treatment, median OS remains around 1 year, as reported in the literature.[7]

When analyzing each of the two second-line chemotherapy regimens, GTX and GmAb, the median OS for the GmAb group was approximately 52 months, which was greater than that of the GTX group, 25 months ($P = 0.029$). The survival obtained in our population was

markedly greater than that published in the literature, particularly for the GmAb group. We checked our results several times to avoid possible errors and obtained the same results. Possible reasons for this high survival may be that our center is a major referral center in the Middle East and North Africa region where patient care may not be afforded by all socio-economic classes. As such, the patient population that seeks medical attention at our center reports the earliest symptoms, thus contributing to a generally earlier diagnosis and better prognosis even when metastatic disease is diagnosed.

There was no statistically significant difference in baseline characteristics and other variables between the two treatment arms, which further suggests that patients who received GmAb may perform better than those who received GTX as second-line therapy after first-line FOLFIRINOX. In a retrospective analysis by Dakik et al., median OS was 22 weeks for patients who received GTX in the second-line setting. However, this analysis did not compare this regimen to other lines of therapy, and first-line therapy was Gemcitabine-based.[10] In a recently published study by Yildirim et al., there was no statistically significant difference between several second-line chemotherapy options, namely Xelox, GmAb, and other regimens (platinum-gemcitabine, FOLFIRINOX, Capecitabine, Xeliri, and FOLFOX), with PFS of 3.2 months, 3.7 months, and 3.5 months, respectively, and with OS of 5.9 months, 5.3 months, and 4.8 months, respectively.[3] Another study by Catalano et al. supported the use of fluoropyrimidine-based second-line chemotherapy for advanced pancreatic cancer, thus confirming the effectiveness and safety, to a greater extent compared with the FOLFIRI regimen, after progression to GmAb.[11] Interestingly, when comparing second-line FOLFIRI and FOLFIRINOX after first-line Gemcitabine-based therapy for locally advanced/metastatic pancreatic cancer at three Italian institutions, the FOLFIRINOX regimen had a favorable toxicity profile and better survival outcomes.[12] Therefore, our study can be considered the first to investigate non-oxaliplatin-containing second-line regimens after first-line FOLFIRINOX.

Furthermore, in our study population, the majority of patients who received either GTX or GmAb regimens did not require dose reduction, with 73.3% and 75.0% of patients from each arm, respectively, not requiring dose modification. However, 26.7% of patients who received GTX required dose reduction starting from cycle one, while only 3.1% of those who received GmAb required dose reduction from cycle one. Moreover, 38.7% of patients who received GmAb did not experience anemia throughout the course of treatment, while the majority of patients who received GTX, 93.3%, had grade I anemia. 60.0% of patients who received GTX had died at the data cut-off time, while 84.4% of patients who received GmAb were still alive. With similar baseline characteristics for both groups, these results support the consideration of GmAb as a better second-line treatment option than GTX, with better tolerance to dose, less anemia, and a better survival profile.

Our study has several strengths and limitations. To our knowledge, it is the first study to investigate non-oxaliplatin-containing second-line regimens after first-line FOLFIRINOX. While our results suggest that GmAb is a better second-line treatment option than GTX, there are several limitations.

Firstly, our sample size was small, which may have contributed to the population not being representative of the general population. Additionally, the retrospective study design is not ideal for assessing OS compared to prospective study designs. While this study design carries a few advantages, such as being suitable for rare diseases like pancreatic cancer progressing on FOLFIRINOX and small patient populations, it has several drawbacks. Retrospective studies are susceptible to selection and memory bias. The study subjects may not be representative of the population, and reasons for non-selection may not be ascertainable. Also, as indicated above, data available in the charts were not collected for research purposes. Therefore, some data may be missing for some patients.

Lack of homogeneity is another concern in a retrospective design. Different people are involved at different times in patient care and data entry, especially when studies look at charts over several years like our study, which spanned 6 years. In addition, prescription bias can exist among our population. Prescriptions may have varied according to patients' risk profiles, and the exact reasons may not have been recorded. Moreover, we cannot determine incidence in a retrospective design, nor can we determine the reason behind loss to follow-up. Reasons for lost follow-ups often cannot be ascertained in retrospective studies and can potentially bias the results.

Conclusion

Our study is one of the few that compare second-line regimens for pancreatic cancer. We have introduced GmAb as a potentially superior second-line treatment option to GTX, with better dose tolerance, less anemia, and a better survival profile. However, larger studies with a prospective design and a larger sample size are needed to confirm this possible difference between the two regimens. In the meantime, we recommend an individualized patient-based approach where either regimen can be considered, taking into account the first-line chemotherapy regimen and performance status. Larger prospective studies are required to better evaluate the differences in outcome and response between the two regimens.

Conflict of Interest

None declared.

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Original Article
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Wire Localization versus Intralesional Methylene Blue Marking for Surgical Excision of Impalpable Breast Lesions

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Abstract

Background: Preoperative marking of impalpable breast lesions is crucial for limiting false negative results and reducing the size of the resected breast tissue, thus improving cosmesis. The aim of this study was to evaluate wire localization versus intralesional methylene blue marking for surgical excision of impalpable breast lesions regarding the success of localization, cost, and limitations of both techniques.

Method: This prospective cohort study included 50 patients with impalpable breast lesions or an area of suspicious microcalcification who were scheduled for surgical excision in the period between June 2020 and December 2021. Patients were randomly allocated into two groups: group I included 25 patients for surgical excision after preoperative ultrasound-guided methylene blue marking. Group II included 25 patients scheduled for surgical excision after preoperative guide wire localization under radiological guidance.

Results: Localization by methylene blue injection has been associated with significantly shorter time of operation with mean duration ($P = 0.018$) and much reduced cost in comparison with guide wire ($P < 0.001$). Postoperative pain, reactions, ecchymosis, accuracy of localization, margin status, and patient satisfaction did not vary significantly between both groups.

Conclusion: Localization by methylene blue injection is not only equally successful to guide wire in locating and identifying impalpable breast lesions for surgical excision, but also is significantly less costly and associated with a shorter duration of operation.

Keywords: Breast neoplasms, Methylene blue, Guide wire, Surgical margin

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Introduction

The widespread use of advanced mammographic techniques has been associated with increased detection of impalpable breast lesions. In the UK, one-third of all breast cancers diagnosed are non-palpable, and even higher rates approaching 50% are

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observed in other developed countries, where breast-screening programs have been implemented, such as the Netherlands.[1, 2] Precise resection of impalpable breast lesions has been a challenge. Preoperative marking is crucial for limiting false-negative results and reducing the volume of breast tissue needed to be excised, thus improving cosmesis.[3, 4] Many techniques have been developed for marking impalpable breast lesions. Intraoperative ultrasound-guided resection has been previously used. Other methods include cryoprobe-assisted localization, clip location, near-infrared fluorescence optical imaging, carbon marking, and radioguided occult lesion localization (ROLL).[5] ROLL was developed in 1998 at the European Institute of Oncology in Milan and has become increasingly popular. ROLL uses a radioisotope, mostly technetium-99m, which is injected intralesionally under radiological guidance preoperatively. The lesion is detected by a gamma probe.[5] Among all localization techniques, guide wire and methylene blue injection have proven the most feasible and effective.[6-8] One of the most commonly used techniques is preoperative guide wire localization (GWL) under ultrasound.[6-8] Despite being effective, this technique has some drawbacks, such as pain during the procedure and wire displacement. The cost of the wire is another issue. Using a technique that is effective, low cost, and easy to learn has been a major concern. Taking the need to decrease the cost into consideration, preoperative intralesional methylene blue injection under ultrasound guidance may be an effective tool. Methylene blue is a readily available and inexpensive dye with a long history of use in humans and minimal side-effects.[9] Another advantage is that it does not affect histologic or immunohistochemical assessment.[10] The aim of this study was to compare ultrasound-guided intralesional methylene blue injection and GWL for surgical resection of impalpable breast lesions regarding: successful localization of the lesion, successful excision of the lesion, incidence of complications, cost, pain, and discomfort.

Patients and Method

Patient selection

This prospective study included 50 patients with radiologically and/or pathologically suspicious impalpable breast lesions or an area of suspicious microcalcification who were scheduled for surgical excision. They were admitted to the Department of Surgery, Medical Research Institute, University of Alexandria, and the Surgical Oncology Unit, Main University Hospital, Faculty of Medicine, University of Alexandria, Egypt. The studied patients were randomly allocated into two groups: group I included 25 patients scheduled for surgical excision of impalpable breast lesions after preoperative ultrasound-guided intralesional methylene blue marking. Group II included 25 patients scheduled for surgical excision of impalpable breast lesions after preoperative GWL under radiological guidance. Patients with pathologically proven malignant breast lesions, a history of allergy, previous breast surgery, and those with impaired renal functions were excluded from the current study.

Ethical considerations

The protocol was approved by the Alexandria University, Faculty of Medicine Ethics Committee before the study started (ethics code: 0106420/2020). The study was explained to prospective patients and written informed consent was obtained before study entry.

Study protocol

All patients included in the study were subjected to thorough history-taking, routine laboratory investigations, full clinical examination with detailed breast examination, ultrasound +/- mammography of both breasts, and ultrasound-guided fine needle aspiration cytology (FNAC) or core tissue biopsy for histopathological assessment. All patients were randomly assigned using a simple closed-envelope randomization technique to two groups at a 1:1 ratio: methylene blue and guide wire groups.

Intralesional methylene blue injection

The injection was performed by an expert radiologist under radiologic guidance of the mass during the immediate preoperative period. Lesions

Table 1. Clinico-demographic criteria of studied patients

	Group 1 (n=25)		Group 2 (n=25)		Total (n=50)	
Age (years)						
Min- Max	29.0-52.0		26.0-51.0		26.0-52.0	
Mean ± SD	40.32 ± 6.848		37.44 ± 6.634		38.88 ± 6.829	
Median ± IQR	40 ± 13		37 ± 9		38 ± 10	
P	*0.138					
Side						
Right	13	52%	15	60%	28	56%
Left	12	48%	10	40%	22	44%
P	##0.569					
Site						
Central	6	24%	4	16%	10	20%
UOQ	9	36%	8	32%	17	34%
UIQ	3	12%	6	24%	9	18%
LOQ	5	20%	5	20%	10	20%
LIQ	2	8%	2	8%	4	8%
P	###0.888					
Size						
Min- Max	10.0-22.0		8.6-22.0		8.6.0-22.0	
Mean ± SD	15.72 ± 3.518		17.834 ± 3.746		17.38 ± 3.747	
Median	15 ± 6		18		18 ± 7	
P	*0.112					
FNAC						
Fibroadenomatoid hyperplasia	5	20%	7	28%	12	24%
Ductal hyperplasia	4	16%	6	24%	10	20%
Duct papillomatosis	9	36%	6	24%	15	30%
Focal epithelial hyperplasia	7	28%	6	24%	13	26%
P	##0.703					
BIRADS						
3	8	32%	7	28%	15	30%
4a	11	44%	10	40%	21	42%
4b	6	24%	8	32%	14	28%
P	##0.819					

P: *P* value for comparing both groups, statistically significant at < 0.05; *Student t-test, ## chi-square test, ### Fisher exact test; IQR: Interquartile range; UOQ: Upper outer quadrant, UIQ: Upper inner quadrant, LOQ: Lower outer quadrant, LIQ: Lower inner quadrant; FNAC: Fine needle aspiration cytology; BIRADS: Breast imaging-reporting and data system

classified as nodules and complex cysts were marked during ultrasonography, while microcalcifications were labeled during mammography. A 1% methylene blue solution was used for all lesions, with a volume of 0.5 ml injected using an insulin syringe and a 26-gauge needle. An additional 0.2 ml was injected during withdrawal of the syringe to mark the tract between the mass and the skin, followed by marking the skin overlying the mass for the site of the incision. The success of the injection should be thoroughly assessed. A completely successful injection is encountered, if the operator can easily identify the mass with discoloration of the mass. A partially successful injection indicates that the surgeon has difficulty identifying the mass, mostly due to the dispersion of the dye in the surrounding tissue. Pain during the procedure should be evaluated as well. Hypersensitivity reactions are reported by radiologists and surgeons.

GWL

GWL is done for all lesions detected by ultrasound. Adequate positioning of the patient is done. The patient takes the supine position, if the lesion is in the inner quadrants; and the supine oblique position, if the lesion is in the outer quadrants; with arms abducted in 90 degrees. The entrance point of the wire is chosen to have the shortest distance to the lesion. Local anesthesia is introduced by superficial injection of lidocaine followed by deeper injection into the tissues surrounding the lesion. The wire is introduced under a real-time guidance along the lateral margin of the transducer visualizing the whole wire during

insertion. The transducer is held in the non-dominant hand and the localization needle held by the other one. Ideally the tip of the wire is positioned 1 cm beyond the lesion. Once well-positioned, the wire is advanced and the needle is withdrawn carefully. For suspicious calcifications and architectural distortion, localization is done under mammographic guidance. Direct 90 degree mediolateral and craniocaudal mammograms are done to assess the position of the lesion. Local anesthesia is introduced. The patients is well positioned standing with her breast horizontally placed on the film cassette and compressed by compression paddles with the craniocaudal film taken. The needle wire is introduced through the hole opposite to the target lesion. Then, compression is applied by paddles in complete medio-lateral oblique view and films are taken. This allows better adjustment of the needle. After localization, two view mammograms are done with wire in position to ascertain good localization. The wire is firmly taped in position with full descriptive report of the localization process. Success of wire localization is assessed by confirmatory post localization 2 view mammogram. The ideal wire localization has to transfix the lesion, pass through its posterior aspect and extend beyond the lesion not more than 1 cm depth. Pain during the procedure should be evaluated as well. Hypersensitivity reactions are reported by radiologists and surgeons.

Surgical excision

All operations are performed under general anesthesia by breast surgeons. In all included patients from both groups, three ml of methylene blue is injected for possible sentinel lymph node biopsy (SLNB) if imprint cytology confirms the marked breast lesion as malignant. Dissection is carried out until the target lesion is reached. Accurate lesion identification is crucial for successful excision. Lesion discrimination relies on the discolored area in the first group (Figure 1) and the end of the wire in the second group (Figure 2). Imprint cytology is used to confirm or exclude malignancy and assess the margin status of proven malignant lesions. If margins are invaded, re-excision is performed to ensure oncological safety. Hemostasis is achieved, and the subcutaneous tissue is closed with absorbable sutures after inserting a drain. The skin is then closed with 3/0 Monocryl. A compression dressing is applied.

The analyzed criteria include:

1. Complete marking of the lesion.
2. Complete excision of the lesion.
3. In cases of malignant lesions proven by imprint cytology, the presence of free margins.
4. Allergic reactions.[5]
5. Difficult lesion identification, characterized by an operative time exceeding one hour from the skin incision.

Complete excision must be thoroughly

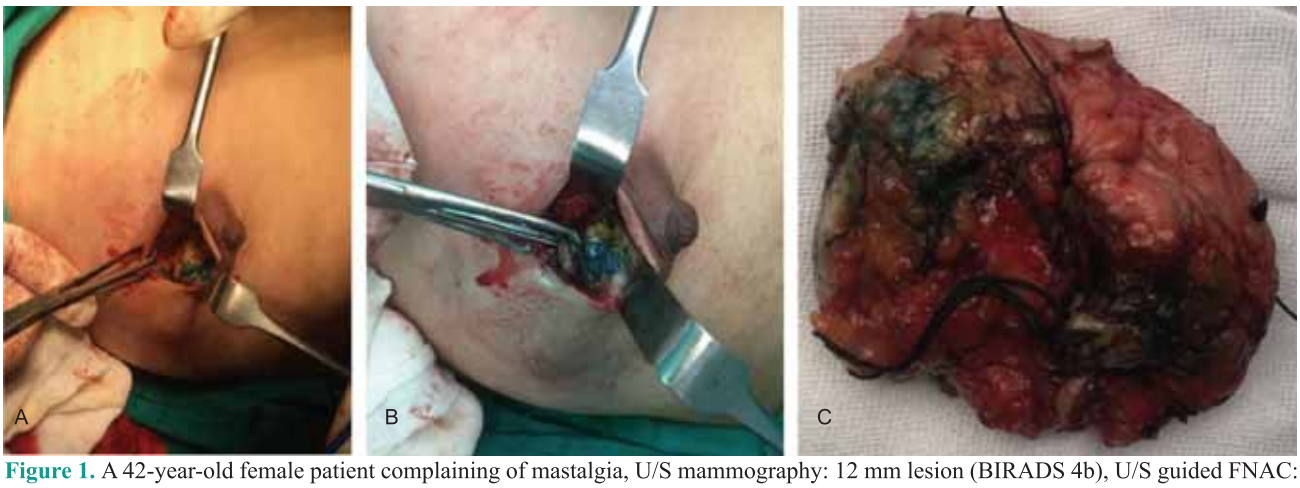

Figure 1. A 42-year-old female patient complaining of mastalgia, U/S mammography: 12 mm lesion (BIRADS 4b), U/S guided FNAC: Focal epithelial hyperplasia, U/S guided methylene blue marking for excision, frozen section: mammary carcinoma with negative margin, SLNB: negative.

BIRADS: Breast imaging-reporting and data system; SLNB: Sentinel lymph node biopsy; U/S: Ultrasound; FNAC: Fine needle aspiration cytology

Table 2. Distribution of the studied patients according to surgical procedure

	Group 1 (n=25)		Group 2 (n=25)		Total (n=50)	
Length of incision (mms)						
Min-Max	15.0-30.0		16.0-30.0		15.0-30.0	
Mean ± SD	21.68 ± 4.25		22.96 ± 4.269		22.29 ± 4.262	
Median ± IQR	20.0 ± 5.0		20.00 ± 5		20 ± 5	
P	#0.332					
Duration of operation in minutes						
Min- Max	55.0- 105.0		60.0- 120.0		55.0- 120.0	
Mean ± SD	77.29 ± 15.60		88.04 ± 16.00		82.78 ± 16.585	
Median ± IQR	72.5 ± 29		85 ± 60		80 ± 25	
P	*0.018					
Imprint cytology						
Benign	16	64%	13	52%	29	58%
Malignant	9	36%	12	48%	21	42%
P	##0.390					
Status of margins in malignant lesions						
Free	9	100%	9	75%	18	85.7%
1 margin invaded	0	0%	3	25%	3	14.3%
2 or more margins invaded	0	0%	0	0%	0	0%
P	###0.284					
SLNB in malignant lesions						
Positive	4	44.4%	4	33.3%	8	38%
Negative	5	55.6%	8	66.6%	13	62%
P	###0.5					
Pain score						
Min – Max	4-7		4-7		4-7	
Mean ± SD	5.32 ± 0.802		5.6 ± .816		5.46 ± .816	
Median ± IQR	5 ± 1		6 ± 1		5 ± 1	
P	#0.193					
Time between technique and operation						
Min – Max	30 -70		720.00 – 1350.00		30-1350	
Mean ± SD	45.60 ± 11.30		966.4 ± 165.65		506.00 ± 479.371	
Median ± IQR	45.00 ± 20		980.00 ± 255.00		395.00 ± 491.25	
P	*<0.001					

P: *P* value for comparing both groups, statistically significant at < 0.05; *Student t-test, # Mann-Whitney test, ## chi-square test, ### Fisher exact test; IQR: Interquartile range; SLNB: Sentinel lymph node biopsy

assessed. For lesions proven malignant by intraoperative imprint cytology, margin status is checked. Free margins indicate complete excision, while invaded margins necessitate enlarging the excised tissue. For lesions not determined as malignant by imprint cytology, complete excision primarily depends on the final histopathological review of the specimen. Slides from all patients are reviewed by an expert pathologist, and any adverse effects on the histopathologic examination should be reported. In cases of pathological discordance, an ultrasound is performed three months after surgery to exclude residual lesions and confirm complete excision.

Pain is assessed subjectively based on a numerical rating scale (NRS). Each patient is asked to provide two pain ratings: one at the time of localization and another for the worst pain experienced during the first 48 postoperative hours. The average of the two ratings represents the patient's pain level. The numerical rating scale ranges from 0 to 10, with zero referring to no pain and 10 reflecting the worst experienced pain.[11]

Statistical analysis of the data

Data were input into a computer and analyzed using the IBM SPSS software package version 22.0 (Armonk, NY: IBM Corp). Qualitative data were described using numbers and percentages. Quantitative data were described using range (minimum and maximum), mean, standard deviation, and median. The Shapiro-Wilk test was utilized to verify the normality of distribution. Significance of the obtained results was judged

at the 5% level.

The mean values of age, size of the lesion, duration of operation, and the time between localization and the start of the operation were calculated and compared across both groups using the independent sample T-test. Length of incision, pain scores, and overall cost of the localization procedure were calculated and compared across both groups using the Mann-Whitney U Test. Side of the lesion, site in relation to the breast, preoperative radiological and pathological findings, frozen section, and margin status were compared across both groups using the chi-square test and Fisher's exact test.

Results

The current study included 50 female patients with radiologically and/or pathologically suspicious impalpable breast lesions or areas of suspicious microcalcifications who were scheduled for surgical excision. They were admitted to the Department of Surgery at the Medical Research Institute of the University of Alexandria, and the Surgical Oncology Unit at

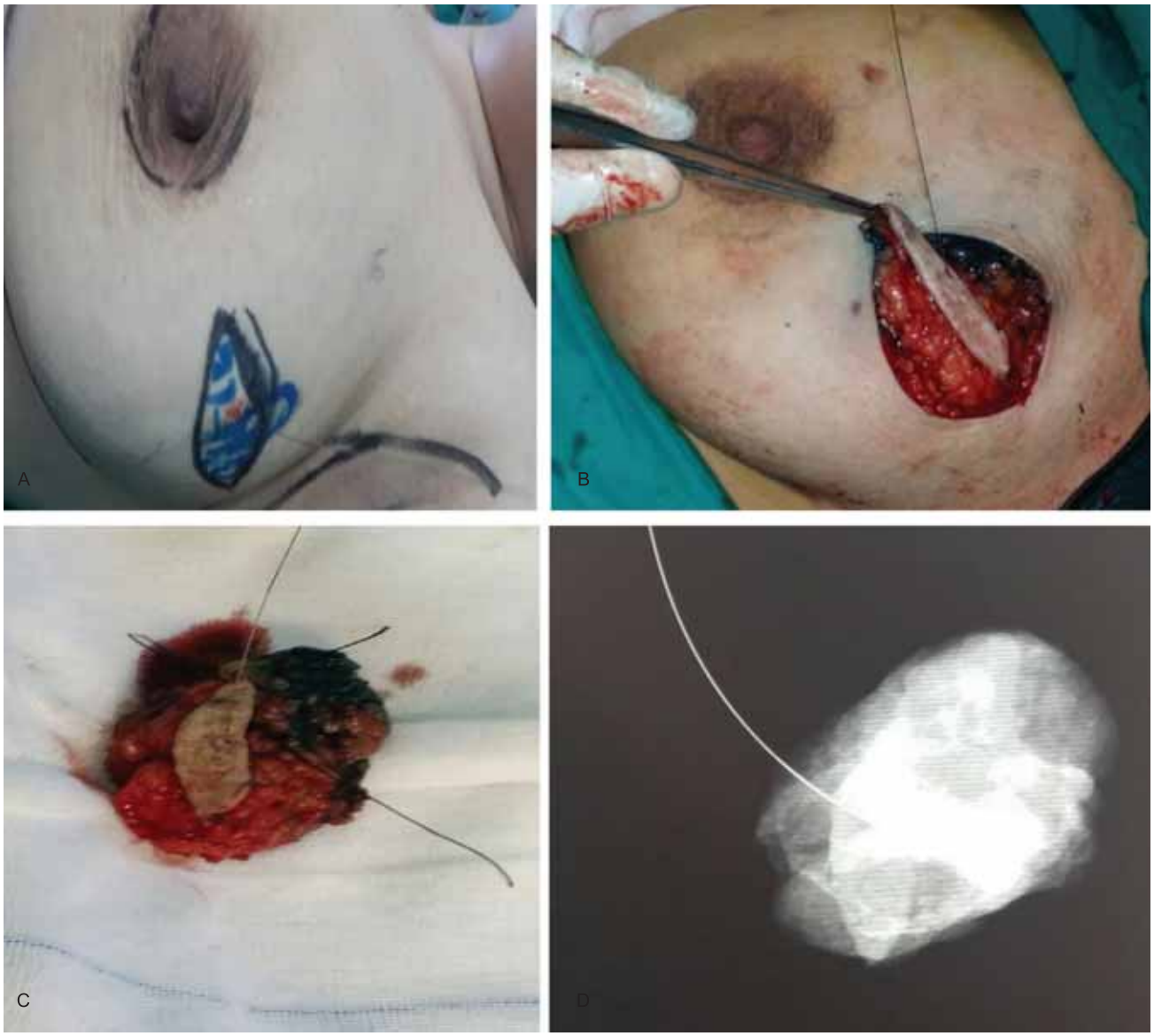

Figure 2. A 38-year-old female patient, U/S mammography: 9 mm lesion (BIRADS 4a), U/S guided FNAC: ductal hyperplasia, GWL for excision, specimen mammography: complete excision, frozen section: fibroadenomatoid hyperplasia.
BIRADS: Breast imaging-reporting and data system; SLNB: Sentinel lymph node biopsy; U/S: Ultrasound; GWL: Guide wire localization; FNAC: Fine needle aspiration cytology

Table 3. Distribution of studied patients according to complications and outcome

Inaccurate localization dislodgement/ dispertion						
Occurred	2	8%	2	8%	4	8%
Not occurred	23	92%	23	92%	46	92%
P	###1.00					
Allergy						
Reaction	1	4%	0	0	1	2%
No reaction	24	96%	25	100%	49	98%
P	###1.00					
Ecchymosis						
Yes	1	4%	5	20%	6	12%
No	24	96%	20	80%	44	88%
P	###0.189					
Patient satisfaction						
Excellent	14	56%	8	32%	22	44%
Good	7	28%	10	40%	17	34%
Fair	3	12%	5	20%	8	16%
Insufficient	1	4%	2	8%	3	6%
P	###0.445					
Cost (Egyptian pounds)						
Min- Max	110-150		700-1020		110 – 1020	
Mean ± SD	126.40 ± 15.780		838.40 ± 96.725		482.40 ± 366.096	
Median ± IQR	120 ± 35		800 ± 185		425.00 ± 680	
P	#<0.001					

*P: *P* value for comparing both groups, statistically significant at < 0.05; # Mann-Whitney test, ### Fisher exact test; IQR: Interquartile range; IQR: Interquartile range

the Main University Hospital of the Faculty of Medicine, University of Alexandria, Egypt, in the period between June 2020 and December 2021. The studied patients were randomly allocated into two groups: group I (GI) included 25 patients scheduled for surgical excision of impalpable breast lesions after preoperative ultrasound-guided methylene blue marking, and group II (GII) included 25 patients scheduled for surgical excision of impalpable breast lesions after preoperative GWL under radiological guidance.

With regard to the distribution of the studied patients based on age, site, side, and size of the lesion, as well as pathological and radiological findings, no significant differences were observed between the two groups, as shown in table 1. Regarding the length of the incision needed for excision of the lesion, there was no significant difference between the two groups ($P = 0.332$). The duration of surgery was shorter in GI, with a significant difference between the two groups ($P = 0.018$), as shown in table 2.

Imprint cytology during surgery detected benign lesions in 16 cases (64%) and malignant lesions in 9 cases (36%) in GI, while in GII, 13 cases were benign (52%) and 12 cases were malignant (48%), with no significant difference between the two groups ($P = 0.390$), as shown in table 2. Out of the 9 lesions proved malignant by imprint cytology in GI, all margins were found to be free in all 9 cases (100%). In GII; however, 3 (25%) out of 12 malignant lesions had one margin invaded and required re-excision of breast tissue related to the invaded margin. The re-excised margins were found to be free in those 3 cases, ensuring the adequacy of re-excision, as shown in table 2.

SLNB in cases proven malignant by imprint cytology was positive in 4 cases (44.4%) and negative in 5 cases (55.6%) in GI, while in GII, 4 cases (33.3%) were positive and 8 cases (66.6%) were negative. The positive cases in both groups were submitted to complete axillary lymph node dissection (ALND), as shown in table 2. Pain was assessed subjectively in patients of both groups.

Top of form

Data were collected on numerical rating scale (NRS) pain scores during the localization

procedure and at five days postoperative. Pain scores were slightly lower in group I, with no significant difference observed between the two groups (P = 0.183). As for the accuracy of localization, in group I, the lesion was found well-stained in 23 cases (92%), and dye dispersion occurred in 2 cases (8%), which hindered the accuracy of localization. This required the surgeon to excise more tissue, but no deformity occurred. In group II, wire dislodgement occurred in 2 cases (8%), affecting the margin status in one of them, while in the other 23 cases (92%), the wire was found well-positioned and fixed, as shown in table 3.

Hypersensitivity reactions were detected in 1 case (4%) in group I, while no reactions occurred in group II. No statistically significant difference was observed between the two groups (P = 1.00), as shown in table 3. Ecchymosis was observed after dye injection in 1 case (4%) in group I, while in group II, ecchymosis related to wire insertion occurred in 5 cases (20%), with no significant difference between the two groups (P = 0.189), as shown in table 3.

All patients were asked about their satisfaction with the technique used and the overall procedure about one month after the operation. Satisfaction was graded as excellent, good, fair, or insufficient. In group I, 14 patients (56%) evaluated the technique and the overall procedure as excellent, 7 patients (28%) reported the technique as good, 3 patients (12%) graded the technique as fair, and 1 patient (4%) was unsatisfied with the procedure. In group II, 8 patients (32%) found the procedure excellent and were completely satisfied with the overall outcome, 10 patients (40%) graded the technique as good, 5 patients (20%) were fairly satisfied, and 2 patients (8%) reported insufficient satisfaction. No statistically significant difference was found between the two groups (P = 0.445), as shown in table 3.

Regarding the cost needed for localization in both groups, it was significantly less costly in group I (110-150 L.E.) than in group II (700-1020 L.E.) (P < 0.001), as shown in table 3.

Discussion

In the current study regarding the overall cost of localization techniques, including the cost of dye/wire and fees for the performing radiologist, localization by methylene blue injection proved significantly less costly than guide wire insertion. Furthermore, localization by methylene blue injection reduced the total time of the surgical operation and provided fairly accurate localization. Accurate localization is judged by the intra-operative identification of the targeted lesion by the performing surgeon, leading to limited breast tissue resection and, subsequently, less breast deformity.[2] Accurate localization is also judged by margin status, if the excised lesion is proven malignant by imprint cytology.

Preoperative intralesional injection of methylene blue and guide wire insertion under radiological guidance are both effective techniques for localizing clinically impalpable suspicious breast lesions for surgical excision. It has always been a significant challenge for surgeons to decide between radical excision and limiting the amount of tissue resection.[2,3] GWL has been the standard of care for a long time in the absence of a better alternative.[2] The use of a wire for preoperative lesion localization was first described by Dodd et al. in 1965.[12] The technique was later modified with the addition of a hooked tip to the wires to aid fixation in situ by Frank et al. in 1976.[13] Despite being the current standard of care for impalpable suspicious breast lesions, GWL suffers from important drawbacks like wire dislodgement and migration, which can, on rare occasions, cause thoracic injuries, kinking, and wire fracture. Technical issues arising intraoperatively include diathermy burns and limitations in incision placement, adversely affecting cosmetic outcomes.[14] Moreover, GWL necessitates the presence of an expert radiologist and takes longer to perform. This rationale mandates that GWL be performed at least several hours or the day before surgery.[13-16] Furthermore, because GWL is advised to be performed on the same day as surgery to prevent migration, scheduling conflicts between the surgeon and the radiologist can occur,

resulting from the need to coordinate multiple procedures on the same day with different teams. Additionally, there is an inability to use wire location in the early morning without causing a significant delay in the operating room.[3, 7, 10] For all these reasons and the increasing number of non-palpable breast lesions detected by ultrasound screening, the need for a rapid and precise alternative has become of great importance.

The blue dye marking offers the advantage of localizing the lesion through direct visualization of the blue area, providing a safe, simple, effective, and low-cost method for localizing non-palpable breast lesions, especially in areas with limited resources.[17] Nasrinossadat et al. found that marking with methylene blue dye is a cost-effective method for localizing impalpable breast lesions.[3] Nasrinossadat et al.[3] concluded that the cost of using metallic wire is four times greater than dye marking, which was confirmed by our results, which showed that the cost of GWL was nearly seven times higher than dye marking. Therefore, we recommend this method as potentially useful for developing countries.

In our study, localization with methylene blue dye has proven to be a cost-effective method for impalpable breast lesions. Intralesional methylene blue injection offers an equally successful localization alternative to guide wires. Drawbacks of this method may include the possibility of excising a larger area than necessary, which may affect the aesthetic outcome, especially in small breasts. This may occur if a longer period passes between injection and excision due to dye dispersion. We attempted to avoid this by minimizing the interval between dye injection and the start of surgery, with a mean interval of 30-70 minutes. Dye dispersion occurred only in two cases (8%), which hindered the accuracy of localization, obligating the surgeon to excise more tissue, but no deformity occurred. This was similar to the results demonstrated by Filho et al.,[16] who used patent blue dye, one of the best dyes for marking impalpable lesions. Patent blue dye diffuses adequately, allowing for safe margins without leading to unnecessary dissection of adjacent tissues.[16-18] However, methylene blue is cheaper and more readily available, especially in developing countries, and its accuracy rate of identification and postoperative complications, including pain, persistent skin staining, and allergic reactions, are comparable to patent blue and charcoal.[18, 19]

Methylene blue localization, in terms of localization accuracy, is comparable to GWL, yet much less costly. Another drawback of dye marking is the inability to perform specimen mammography after excision, which can be done using the GWL method to ensure complete removal of the mass. Athanasiou et al. reviewed 18 randomized controlled trials with 3,112 patients comparing different techniques for localizing impalpable breast lesions[20] and concluded that all other techniques were equivalent to GWL in terms of successful excision, localization complications, operative time, and overall complications.[19]

Another drawback of using dye for localization is the incidence of allergic reactions, which have been reported to occur in 0.06 to 2.7% of cases, with an average value of 0.71%.[17] In the current study, only one patient (4%) experienced a mild allergic reaction. One study suggested that the incidence of allergic events is mainly related to SLNB, which requires a larger volume of dye, usually 2 to 4 ml.[20] However, the volume of methylene blue used for marking non-palpable lesions is 0.2 mL,[21] but this conclusion requires further investigation. In all included patients in both groups, 3 ml of methylene blue were injected for possible SLNB, if imprint cytology indicated that the marked breast lesion was malignant. Only one patient experienced an allergic reaction to the dye, but we still strongly recommend having hydrocortisone injections and epinephrine available at the radiology center where dye injections are performed in case of rare reactions. Methylene blue has an acceptable, relatively low rate of allergic reactions, which is significantly outweighed by its low cost and accurate localization rates.

The SAVI SCOUT® guidance system has been approved by the U.S. Food and Drug Administration since 2014.[22] Briefly, a non-

radioactive infrared (IR)-activated electromagnetic wave reflector is placed into the breast under radiological guidance. The reflector is often placed under ultrasound or mammographic guidance, and an audible signal from the implanted reflector is then detected using the manufacturer's handpiece-and-console system.[23] Falcon et al. concluded that the SAVI SCOUT guidance system is comparable to guide wire localization and could achieve successful localization rates in up to 97% of cases,[24] similar to the 90 to 100% reported success rates for wire, RSL, or SCOUT.[25] The failed localizations were almost entirely due to technical defects. Compared with wire localization, a cited potential disadvantage of the reflector is the inability to move or retrieve it once deployed. The original SAVI SCOUT® console was approved to detect reflectors placed up to 5 cm in depth,[24] which is a drawback for deeply seated lesions. A major advantage is that the SAVI SCOUT® reflector was approved for up to 30 days of implantation.

Our prospective cohort study compared intralesional methylene blue injection for localizing breast lesions to one of the most successful techniques, GWL. It demonstrated a statistically significant preference for dye injection over guide wire in terms of cost and operation time. Moreover, it provided equally successful localization, raising the possibility of using dye injection as an alternative. Although we can conclude that localization by methylene blue injection is as successful as GWL for impalpable breast lesions requiring surgical excision and is significantly less costly and associated with shorter operation durations, this study has one important limitation: it could not be applied to non-ultrasound-detected impalpable lesions. We cannot conclude the feasibility of intralesional dye marking under mammographic guidance, which requires a more skilled surgeon. Therefore, we recommend further studies to confirm our findings with a larger volume of cases.

Conclusion

Preoperative marking of impalpable breast lesions is crucial for limiting false negative results and reducing the size of the resected breast tissue, thereby improving cosmesis. Localization by methylene blue injection is not only equally successful as the guide wire in locating and identifying impalpable breast lesions for surgical excision, but it is also significantly less costly and associated with a shorter duration of operation.

Conflict of Interest

None declared.

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Original Article
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Prostate Cancer Survival Analysis of 872 Patients in Southern Iran: A Retrospective Cohort Study

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Abstract

Background: Prostate cancer remains one of the most common and lethal cancers among men worldwide. This study aimed to investigate the characteristics, prognostic factors, and outcomes of patients with prostate cancer who were treated and followed up in Shiraz, southern Iran over the past 12 years.

Method: This retrospective medical chart review was performed on 872 patients with prostate cancer who were treated and followed up in the Radiation Oncology Department of Shiraz University of Medical Sciences. The survival analysis was conducted for the patients, and the receiver operating characteristic (ROC) curve analysis was performed for the prostate-specific antigen (PSA) level.

Results: The median age of the patients at presentation was 69 years (range 35-91 years). In terms of local treatments, 28% of the patients underwent prostatectomy, and 23% were treated with transurethral resection of the prostate. The remaining 49% of patients were treated with non-surgical therapies. Patients between 55 and 75 years had the longest survival duration. The shortest survival was observed in the third Gleason group and those over 75 years old, while the first Gleason group and patients younger than 55 years had the longest survival duration. Hypoalbuminemia had no effect on the survival duration. A PSA level of 33.8 ng/dl was the most suitable cut-off point to predict bone metastasis, and patients with a PSA level of more than 33.8 ng/dl had significantly less survival duration than the others.

Conclusion: More aggressive treatment and shorter follow-up intervals are recommended for patients with an initial PSA level of more than 33.8 and those younger than 55 years old.

Keywords: Prostatic neoplasms, Survival analysis, Prostate-specific antigen, Prognosis, ROC curve

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Introduction

Based on the global cancer report, prostate cancer is the third most common cancer and the fifth leading

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cause of male mortality in recent years.[1] It is primarily a cancer of the elderly, with more than 75% of cases occurring in patients older than 65 years of age.[2] Typically, patients present with lower urinary tract symptoms that are not significant for this diagnosis. In fact, the European Association of Urology recommends screening men with at least 10 to 15 years of life expectancy in order to detect more localized disease.[3]

Prostate-specific antigen (PSA), a protein specific to prostate tissue, is measured as an indicator of prostate function. According to the latest report from the National Cancer Institute, the age-standardized incidence rate of prostate cancer has increased, while the mortality rate has decreased due to early tumor detection by checking PSA levels.[4] Another way to screen for prostate cancer is digital rectal exam (DRE). Regardless of the PSA level, patients with palpable nodularity or asymmetry of the prostate gland in DRE undergo transrectal ultrasonography (TRUS) and biopsy.[5-7]

Histologically, there are four types of prostate cancer: adenocarcinoma (the most common type), transitional cell carcinoma, small cell carcinoma, and sarcoma.[8] The Gleason score is the most widely accepted grading system for prostate cancer. In this scoring system, the PSA level and clinical staging are crucial for deciding on the proper treatment.[8, 9] Treatment options for these patients include prostatectomy, radiotherapy, hormone therapy, and orchiectomy. Recent studies have shown that overall survival in prostate cancer is affected by the site of metastasis and pre-operation albumin and PSA levels.[10-13] Early initiation of anti-androgenic medication along with fewer sites of metastasis is suggested as an effective factor for increasing prostate cancer survival.[14]

Prostate cancer is the sixth most common cancer in Iran, with a higher incidence compared with other Asian countries.[15, 16] Due to the lack of sufficient national evidence about the survival of prostate cancer in southern Iran, we conducted a study on overall survival and associated factors of prostate cancer during a 12-year survey at Shiraz University of Medical Science.

Materials and Methods

In this retrospective cohort study, we included 872 men with proven invasive prostate adenocarcinoma who referred to the Shiraz

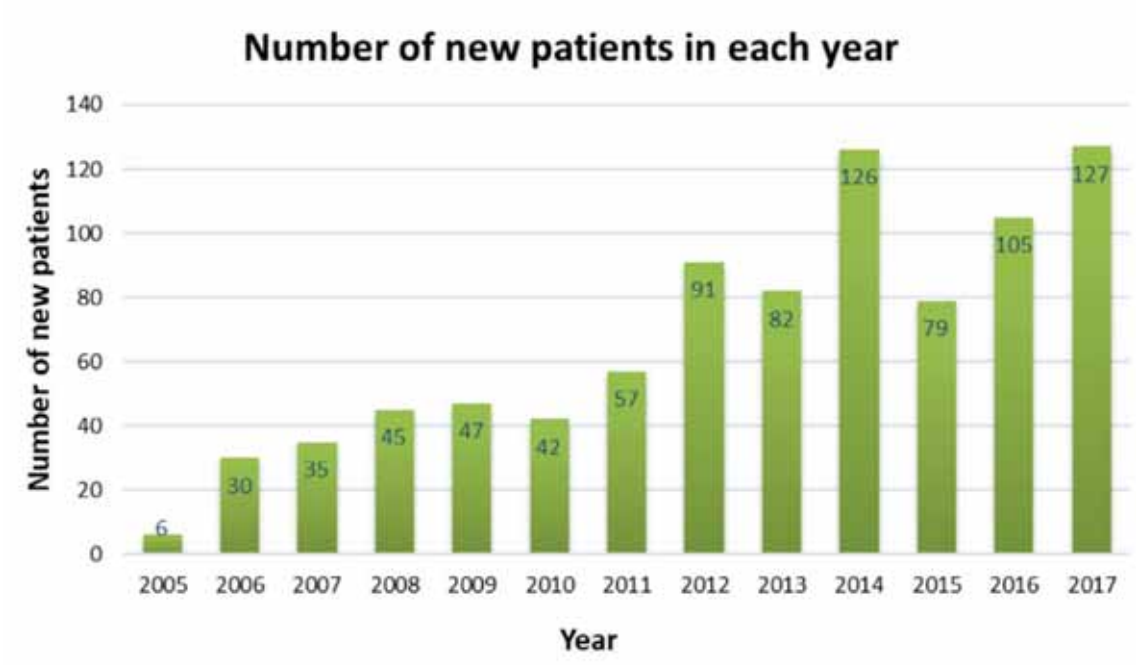

Figure 1. This figure shows the distribution of patients diagnosed during the study period.

Table 1. Multivariate analysis of prognostic factors for overall survival

Variables	Crude HR ^-1 (95% CI)	*P*-value	HR ^-1 adjusted (95% CI)	*P*-value
Age (years)				
<55	ref	< 0.001		0.647
55-75	0.512 (0.232 - 1.132)	0.098	0.000 (0.000-0.000)	0.989
>75	0.418 (0.291 - 0.601)	< 0.001	0.447 (0.082-2.427)	0.351
Grade Group				
1	ref	0.001		0.243
2	0.411 (0.240-0.701)	0.001	0.648 (0.078-5.348)	0.687
3	0.391 (0.213-0.719)	0.003	0.682 (0.065-7.185)	0.750
4	0.871 (0.436-1.740)	0.695	5.237(0.402-68.151)	0.206
5	0.438 (0.230-0.832	0.012	0.712 (0.061-8.240)	0.785
PSA (ng/ml)		0.001		0.019
<33.8	ref		ref	
≥33.8	0.164 (0.109-0.245)		0.191 (0.047-0.765)	
Surgery type				
No surgery	ref	0.001	ref	0.218
TURP	2.257 (1.466-3.474)	< 0.001	4.451 (0.798-24.813)	0.089
prostatectomy	2.369 (1.375-4.080)	0.002	1.294 (0.157-10.676)	0.811
Hormone therapy		0.002		0.034
No	ref		ref	
Yes	2.024 (1.302-3.145)		6.277 1.144-34.439)	
Orchiectomy		0.001		0.007
No	ref		ref	
Yes	0.301 (0.183-0.497)		0.145 (0.036-0.588)	
Albumin (g/dl)		0.838		0.501
<4	ref		ref	
≥4	0.895 (0.309-2.595)		1.938 (0.282-13.325)	
Radiotherapy		0.001		0.281
No	ref		ref	
Yes	0.895 (0.309-2.595)		2.849 (0.425-19.085)	
Chemotherapy regimen				
No chemotherapy		<0.001		
CTX + VCR	0.042 (0.009-0.188)	<0.001		
Docetaxel	0.309 (0.065-1.466)	0.139		0.079
CTX + VCR + Docetaxel	0.115 (0.010-1.280)			
Others	0.310 (0.063-1.532)	0.151		
Unknown	0.444 (0.090-2.190)	0.319		

CI: Confidence interval; PSA: Prostate-specific antigen; HR: Hazard ratio; TURP: Transurethral resection of the prostate; CTX: Cytoxan (cyclophosphamide); VCR: Vincristine

University of Medical Sciences Radiation Oncology department from January 2005 to December 2017. The study was approved by the Ethics Committee of Research of Shiraz University of Medical Sciences (IR.SUMS.MED.REC.1401.107), and all patients signed informed consent. Non-invasive prostate cancer, such as "carcinoma in situ," and pathologies other than adenocarcinoma, such as lymphoma and sarcoma, were excluded. A data gathering form was designed under the supervision of two Radiation Oncologists. The Radiation Oncology department was the only center for non-surgical treatments of prostate cancer in the Fars province during the study. These Radiation Oncologists taught two last-year medical students about the nature of the disease, its treatment, and how to retrieve data from clinical records. The medical students gathered the demographic and clinico-pathological information from the clinical records and wrote them down in the data sheet. Retrieved data was rechecked by Radiation Oncologists at the end.

The variables included in the study were age at incidence, Gleason sum and stage, histology, serum hemoglobin (Hb), albumin (Alb), and PSA, number and ratio of positive biopsies, type of surgery, chemotherapy regimen, medical or surgical (orchiectomy) hormone therapy, radiation dose, and metastasis. The time of the pathology

report proving prostate cancer was assumed as the disease presentation time. Patients were classified into three groups based on their age at presentation: under 55 years old (early prostate cancer), 55-75, and over 75 years old. Hypoalbuminemia was defined as an Alb level less than 4 g/dl. Hb under 14 g/dl was assumed as anemia, and Hb more than 18 g/dl was assumed as polycythemia. The chemotherapy agents used were cyclophosphamide (CTX), vincristine (VCR), and Docetaxel. In this study, and according to the pathologic report, the Gleason grade groups were applied for defining tumor grade and risk of tumor recurrence. The Gleason grade group scoring system included the scores of Gleason grade and Gleason pattern.

The data was analyzed using SPSS version 23. Each patient had its own specific ID, and the data was checked to omit any repeated cases. Before all parametrical tests, a Kolmogorov-Smirnoff normality test was done, and if the variable had no normal distribution, non-parametric tests were done instead. In all statistical tests, the maximum acceptable amount of type one error was assumed to be 0.05. To describe quantitative variables, mean and median were used as central tendency measures, and standard deviation (SD) was used as the dispersion index. T-tests were used to compare quantitative variables in different subgroups. Kaplan-Meier survival plots were drawn for categorized Alb and PSA levels, age, and grade groups. Tarone-Ware test was done to compare survival in the mentioned subgroups. Receiver operating characteristic (ROC) curve analysis was done to determine the most suitable PSA cut-off point to predict further

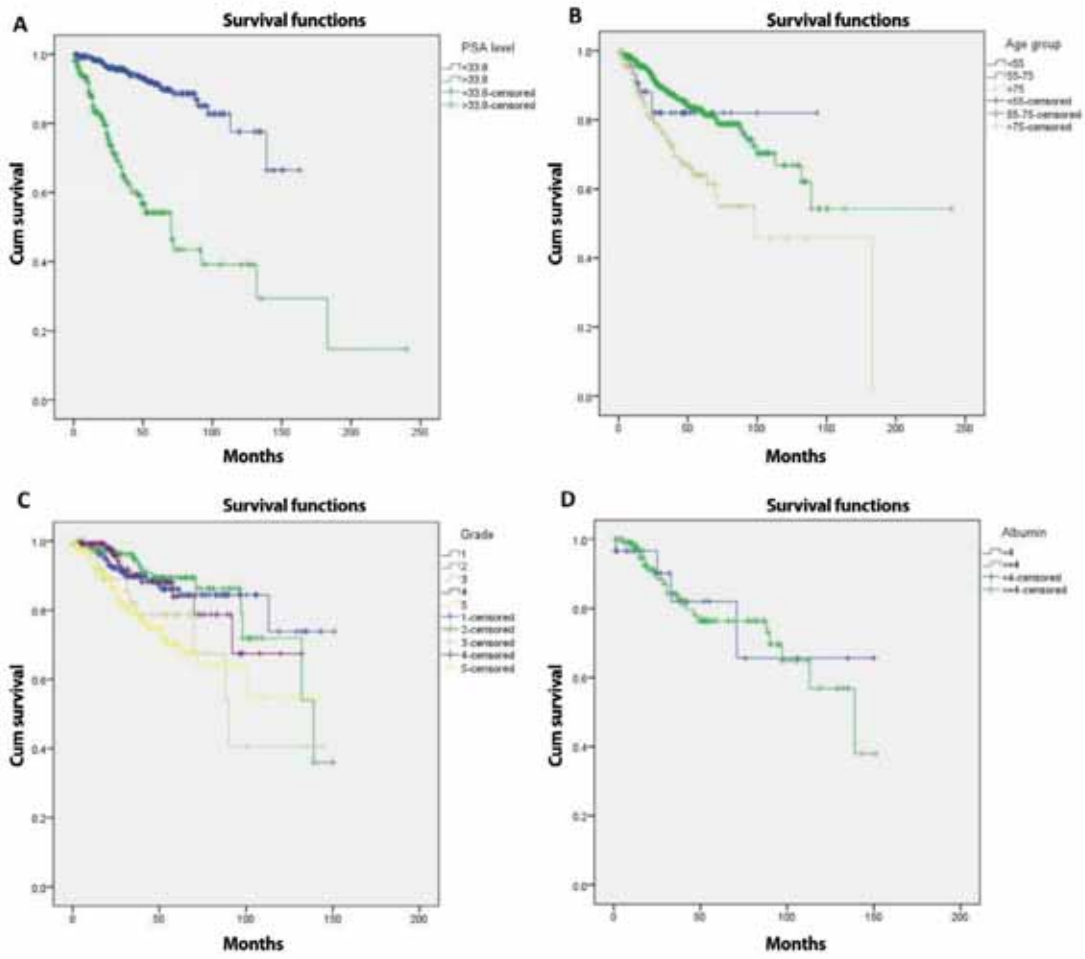

Figure 2. This figure shows the prognostic impact of the serum PSA level (A), age (B), tumor grade (C) and serum albumin level (D) on the overall survival rate of 872 patients with prostate cancer.
PSA: Prostate-specific antigen; Cum: Cumulative

bone metastasis. Finally, Cox regression analysis was used to determine the possible effect of age, grade group, PSA and Alb level, surgery type, hormone therapy and orchiectomy, radiotherapy, and chemotherapy regimen on patients' survival. Crude and adjusted hazard ratios were calculated for these factors.

Results

The total number of patients was 872, of whom 783 were eligible for survival analyses. The frequency of cases per year is shown in figure 1. The median age of the patients at presentation was 69 years (range 35-91 years). In terms of local treatments, 28% of the patients underwent prostatectomy, and 23% were treated with transurethral resection of the prostate (TURP). The remaining 49% of patients were treated with non-surgical therapies. Radiotherapy was prescribed for 756 patients during their treatment, with a mean radiation dose of 54 Gy. 708 patients received single-agent or multiple-agent hormone manipulation during the course of treatment. The differential frequency of each single agent was as follows: Bicalutamide for 9 patients, Zoladex for 152 patients, Cyprotroneacetate for 84 patients, Dipherline for 449 patients, Eligard for 70 patients, Flutamide for 200 patients, Decapeptyl for 203 patients. In addition, 56 patients underwent orchiectomy in their treatment course. Zoledronic acid was prescribed for 122 patients with skeletal metastasis. The most frequent Gleason grade groups were 5 and 1, and the least frequent grade group was 3.

The mean Hb level among all the patients was 12.3 g/dl, and 74.5% of patients were anemic, with less than one percent of them having polycythemia. The Hb level among our patients [12.3 (95% CI 1.43-1.94) g/dl] was significantly lower than 14 g/dl. The mean serum Alb level was 4.2 ± 0.46 g/dl. Hypoalbuminemia in these patients had no effect on the survival duration (Table 1). The mean PSA level was 94.7 and 21.7 in the positive and negative bone scan groups, respectively ($P < 0.001$). There was no difference between the two groups in Hb and Alb. Among the 872 patients, 386 patients had a positive bone scan during their follow-up. Patients with a positive bone scan had significantly higher numbers of positive prostate biopsies ($P < 0.001$). The Kaplan-Meier survival curves of the age group, grade group, PSA level, and serum Alb level are illustrated in figure 2.

In multivariate survival analysis, PSA level ($P = 0.019$), hormone therapy ($P = 0.034$), and orchiectomy ($P = 0.007$) were independent predictors of overall survival (Table 1). A ROC curve was applied for the PSA cut-off point to predict skeletal metastasis. The AUC (Area under the curve) for PSA level predicting bone metastasis was 0.709 (Figure 3).

Discussion

In our study of 872 cases of prostate cancer, the median age at presentation was 69 years. More than 80% of our patients underwent hormone therapy, and three-quarters of them were anemic. A higher PSA level was a strong predictor of lower survival but more bone metastasis. In multivariate analysis, PSA level, hormone therapy, and orchiectomy independently predicted the patients' survival.

The frequency of patients referred here from 2005 to 2017 had an increasing trend. There was an increasing incidence of prostate cancer in 15 countries all around the world in the last two decades of the past century.[17] This increasing trend could be due to both the increasing incidence of prostate cancer in our region, improvement of diagnostic methods, and higher clinical suspicion.

The median age of the patients referred to our clinic was 69 years. In Polyakov et al.'s study on 1564 patients with prostate cancer published in 2017, the median age at presentation of disease was 71 years.[18] These findings are relatively compatible with our study.

In the current study, 55 to 75-year-old patients had the longest overall survival. Age alone has no significant effect on survival in multivariate analysis; therefore, the observed survival difference in age groups may be attributable to other factors. In a Brazilian study, age was not an independent prognostic factor for prostate cancer when the effect of other variables was

omitted.[19]

In Grönberg et al.'s study, done on more than 6000 patients, there was no significant difference in survival between different age groups; however, a higher rate of high-grade disease in younger patients was observed.[11] Longer survival time in the 55-75 year age group may be due to more aggressive disease in younger patients and a higher rate of comorbid diseases in elders. Because of the shorter survival and higher rate of high-grade disease in younger patients, a more aggressive approach and more frequent follow-ups are recommended for them.

In the present study, the mean PSA level was significantly higher in patients with further bone metastasis, and those with PSA > 33.8 ng/dl had a lower survival rate. In a study on 1873 patients, PSA levels between 20 and 70 had a reverse correlation with survival duration; however, in PSA levels between 70 and 100, there was no relationship with survival.[12] As a matter of fact, in patients with higher initial PSA, a more aggressive approach to detect early bone metastases is needed.

Low Alb levels and patients' anemia had no significant effect on survival duration. The mean Hb level was significantly lower than the normal level. In Sejima et al.'s study published in 2013 on 179 patients, lower levels of Alb before surgery were significantly related to higher rates of disease recurrence.[13]

In a study in 2018, pretreatment Alb/globulin was an independent prognostic factor for progression-free survival.[20] In many studies, there is a relationship between cancer and anemia[21, 22] and anemia makes cancer patients' prognosis worse.[23] Further studies with larger sample sizes are needed to assess the effect of Alb level and Hb on survival.

The strengths of our study were the large sample size and long-term follow-up to determine prognostic factors of prostate cancer and define PSA level as a predictor of bone metastasis. The limitations of our study were retrospective design,

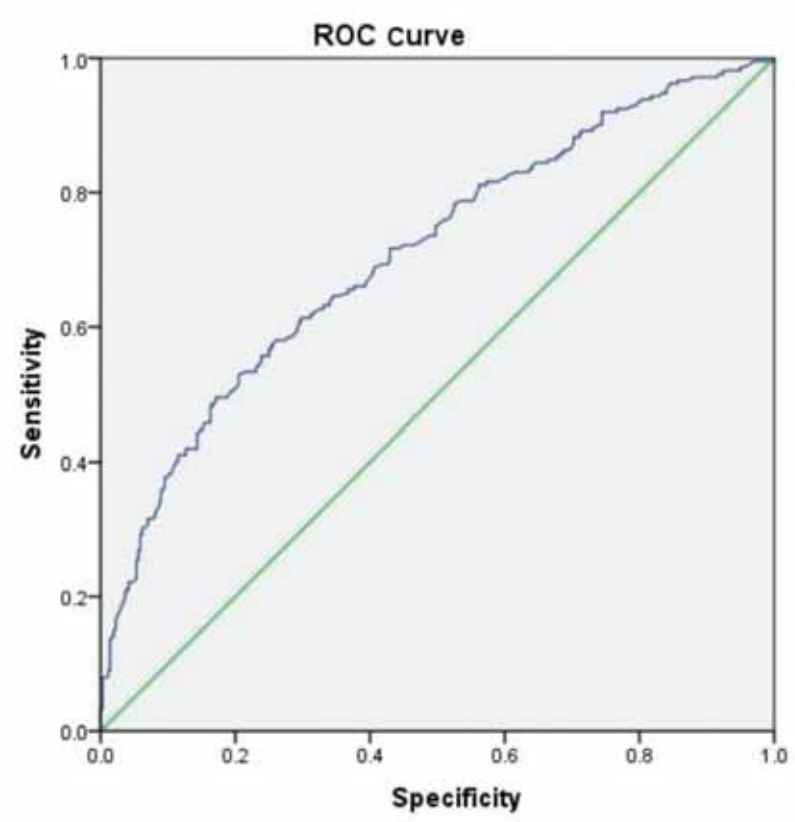

Figure 3. This figure shows the ROC curve for PSA cut-off point to predict skeletal metastasis.
ROC: Receiver operating characteristic; PSA: Prostate-specific antigen

lack of uniform pathological reports, and missing data.

Conclusion

Based on our findings, we observed higher levels of aggression in the behavior of the disease in patients who are under 55 years old, emphasizing the importance of early screening. However, larger prospective studies are needed in our society to determine the appropriate age for starting screening and the PSA level that predicts a clinically significant form of the disease.

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Conflict of Interest

None declared.

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Case Report
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Disseminated Metastasis after Resection of Sacrococcygeal Teratoma with Mucinous Adenocarcinoma: A Case Report

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Abstract

Mature teratoma is a common tumor that can undergo malignant transformation, either in ovarian or extragonadal sites. While adenocarcinoma superimposed on sacrococcygeal teratoma is rare, mucinous variants have been reported in only five cases. Here, we present a case of a young girl with disseminated metastasis of mucinous carcinoma, initially of unknown primary origin. Further investigation by a dedicated multidisciplinary team (MDT) revealed a focus of mucinous carcinoma (intestinal type) in a sacrococcygeal teratoma incompletely resected five years earlier. The patient is currently undergoing second-line chemotherapy after experiencing side-effects on the first-line regimen. Pathologists, gynecologic oncologists, and surgical oncologists should exercise caution when dealing with locally aggressive teratomas, thoroughly searching for malignant components and conducting short-term follow-up.

Keywords: Teratoma, Adenocarcinoma, Mucinous, Unknown primary, Sacrococcygeal

Introduction

Mature teratoma of the ovary is the most common neoplasm, representing 20% of all ovarian tumours. Although rare, malignant transformation of teratoma has been described in 1%-2% of cases.[1] The commonest associated malignancy was squamous cell carcinoma, adenocarcinoma and carcinoid tumour, in this order.[2, 3] In addition, it has been recognized that the prognosis of carcinomas associated with teratoma is worse than that of epithelial ovarian carcinomas, regardless of whether postoperative chemotherapy or radiotherapy is given. This may be explained by the

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rarity of this tumour, posing a significant challenge to developing standardized adjuvant management protocols.[4]

Sacrococcygeal teratomas are relatively uncommon, and most are generally present since birth. However, when sacrococcygeal germ cell tumours are predominantly intrapelvic, they may be asymptomatic for years and are at risk for malignant transformation.[5]

We herein report what might be the sixth case in literature of malignant transformation arising in a sacrococcygeal teratoma.

Case Presentation

A 22-year-old female patient presented with left supraclavicular, left pelvic, and upper thigh swellings to the outpatient clinics of the Oncology Center at Mansoura University in late December 2019. There was no medical history, but the patient had undergone excision of a cystic teratoma in the sacrococcygeal region five years ago. Revising the computed tomography (CT) dating back to 2014 of this lesion, the CT showed a well-defined large hypodense cystic lesion in the presacral space posterior to the rectum, compressing and displacing it anteriorly and contacting the sacrum posteriorly, measuring 14 × 13.5 × 11 cm. The lesion also showed a small projection in its posterolateral wall with fine calcifications. Additionally, post-contrast magnetic resonance imaging (MRI) showed a well-defined lesion in the presacral region, mainly cystic, measuring

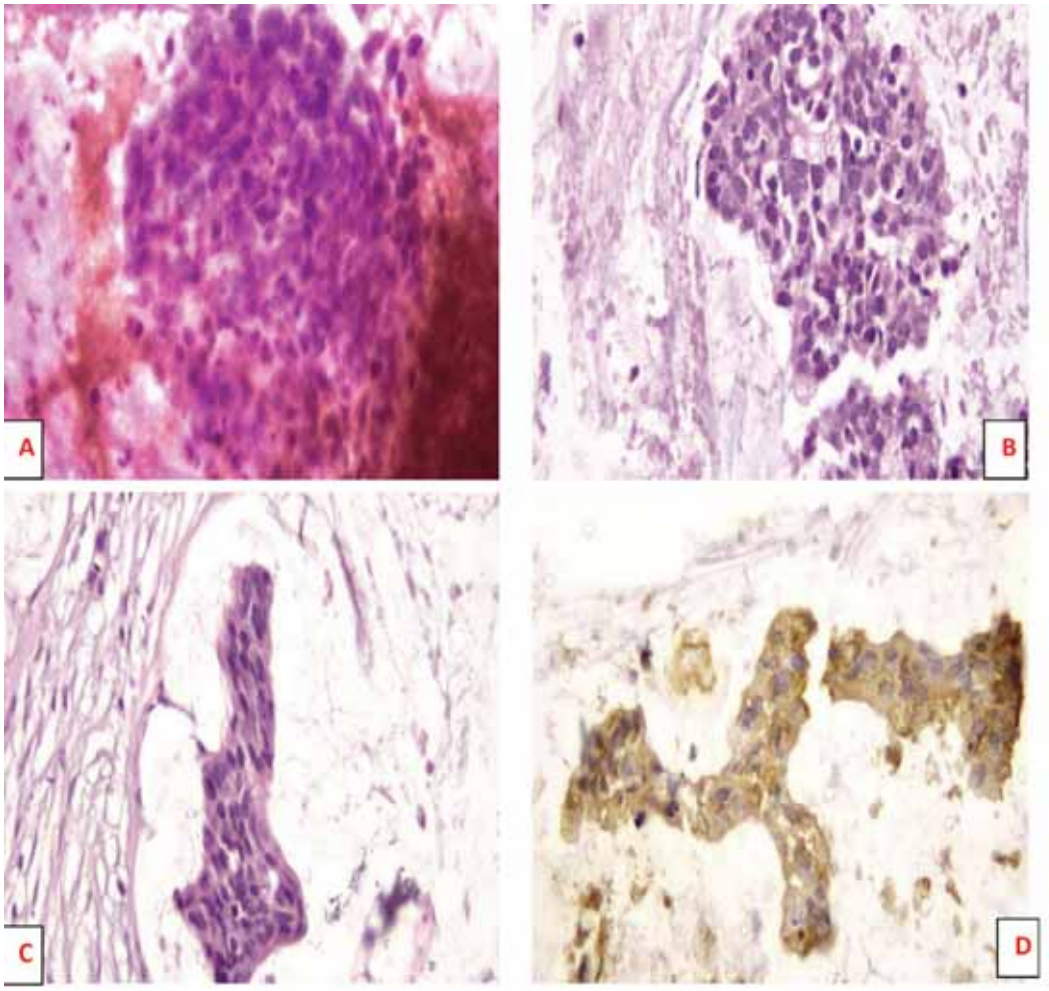

Figure 1. Fine needle aspiration cytology from a cervical node showing aggregates of malignant cells (A, smear, H&E, 40×) with associated pools of mucin (B, cell block, H&E, 40×). Histopathologic examination of the true-cut biopsy of an iliac mass showed neoplastic cells floating in pools of extracellular mucin (C, H&E, 40×). Immunohistochemical expression for CK20 was detected in tumor cells (D, H&E, 40×).

15 × 12.5 × 11.5 cm with a small solid component measuring 4 × 3.5 × 3.3 cm with the same relations described in the CT. The lesion was incompletely excised with extensive bleeding from the presacral plexus, leaving residue on the sacrum five years ago. The post-resection pathological analysis revealed a benign cystic teratoma with atypical epithelial proliferation within the mucoid background (no further immunohistochemistry (IHC) was done at that time).

In December 2019, the patient came because of neck swelling, and ultrasonography revealed suspicious lymph nodes on the neck in the left lower deep group, irregular in shape with lost hilum, measuring 2.6 × 1.6 cm. Non-contrast pelvic and upper thigh MRI showed a large soft tissue lesion displacing the intestinal loops and the urinary bladder, extending to the upper abdomen superiorly and inferiorly to the upper left thigh through the left obturator foramen, with the possibility of recurrence of the previous mass or a malignant left ovarian mass.

Fine needle aspiration cytology (FNAC) from the suspicious left deep cervical lymph node showed a malignant smear of metastatic mucoid adenocarcinoma (Figures 1A and 1B). Core needle biopsy from the left iliac mass showed a picture compatible with invasive mucinous carcinoma that showed a positive immunohistochemical reaction for CK20 with a negative reaction for CK7 and CDX2 (Figures 1C and 1D). The tumor was MSI stable/MMR proficient.

Tumor markers were as follows: CA-125 = 17.05, CA-19-9 ≤ 0.6, and carcinoembryonic antigen (CEA) = 113, where CEA was later raised to 147 while on chemotherapy.

Three months later, a CT scan showed generalized lymphadenopathy, bilateral external and internal iliac, inguinal, and abdominal lymph nodes measuring 3.8 × 2.4, 2.5 × 2.3, and 3.5 × 2 cm, respectively, with amalgamated lymph nodes in the left lower deep cervical and supra-clavicular region measuring 4.5 × 2.3 cm. Additionally, multiple bilateral pulmonary nodules, the largest

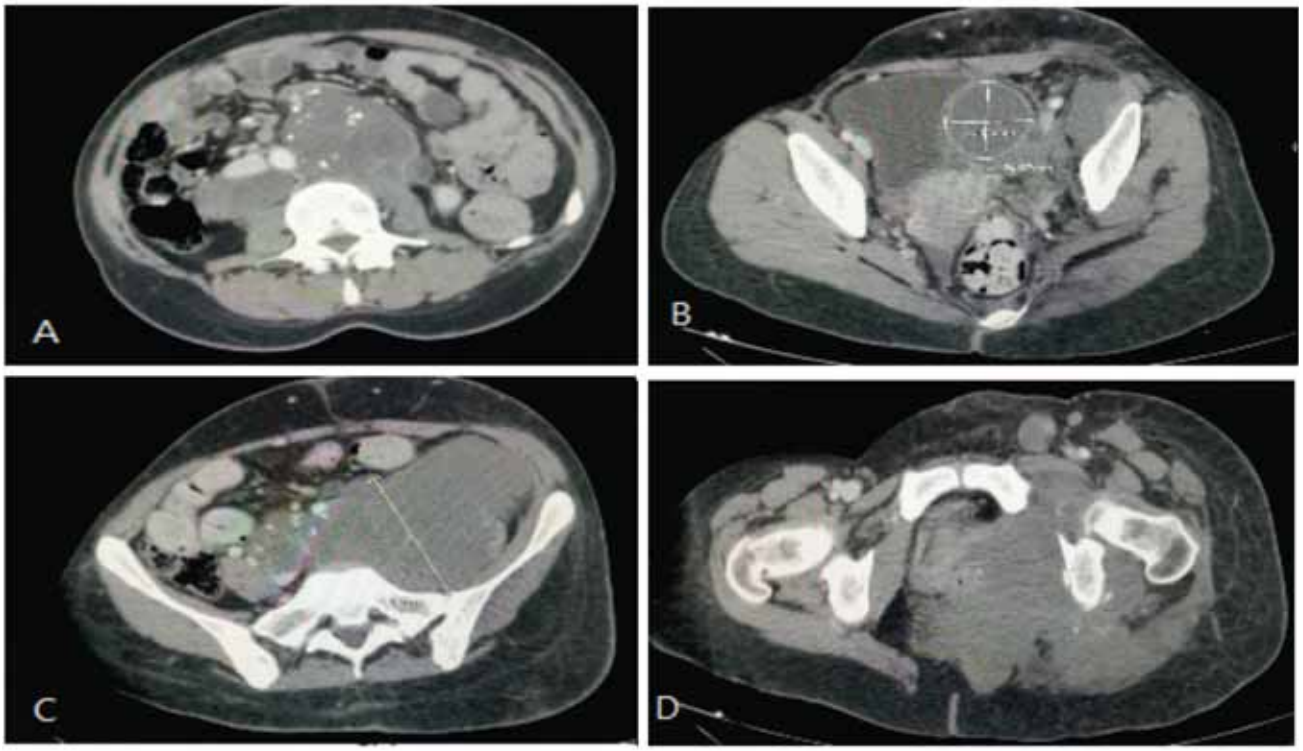

Figure 2. Computed tomography scan showing A) a hypodense soft tissue retroperitoneal mass in the pelvis with foci of calcification, B) a mass inseparable from the bladder, indenting its left wall, C) a mass inseparable from the left ilio-psoas muscle, and D) another similar mass seen in the left side of the pelvic wall and left peritoneal region, inseparable from the endocervix, ischial and pelvic bones, and inseparable from the left gluteus, obturator, and left levator-ani muscles.

being 9 mm on the left lower lobe, were identified, along with a hypodense soft tissue retroperitoneal mass that was inseparable from the left ilio-psoas muscle, encasing the lower abdominal aorta, its bifurcation, and left iliac vessels, showing foci of calcification and measuring 17.9 × 11.4 × 8.6 cm. Another similar mass was seen in the left side of the pelvic wall and left peritoneal region, inseparable from the internal cervix, ischial and pelvic bones, inseparable from left gluteus, obturator, and left levator-ani muscles, measuring 13.2 × 12.7 × 9.5 cm. The masses were associated with mild to moderate left hydronephrosis (Figure 2 A-D).

The patient was treated as a case of metastasis of unknown origin (MUO) of probably gastrointestinal tract (GIT) origin with XELOX for three cycles, where a sensitivity reaction to oxaliplatin developed, and the patient refused to continue. Thereafter, the patient was shifted to weekly paclitaxel + carboplatin as a case of metastasis of unknown primary of germ cell tumor origin, as the patient clinically progressed on XELOX and CEA levels increased.

Follow-up CT scans showed a stationary course on chemotherapy. Considering the stationary

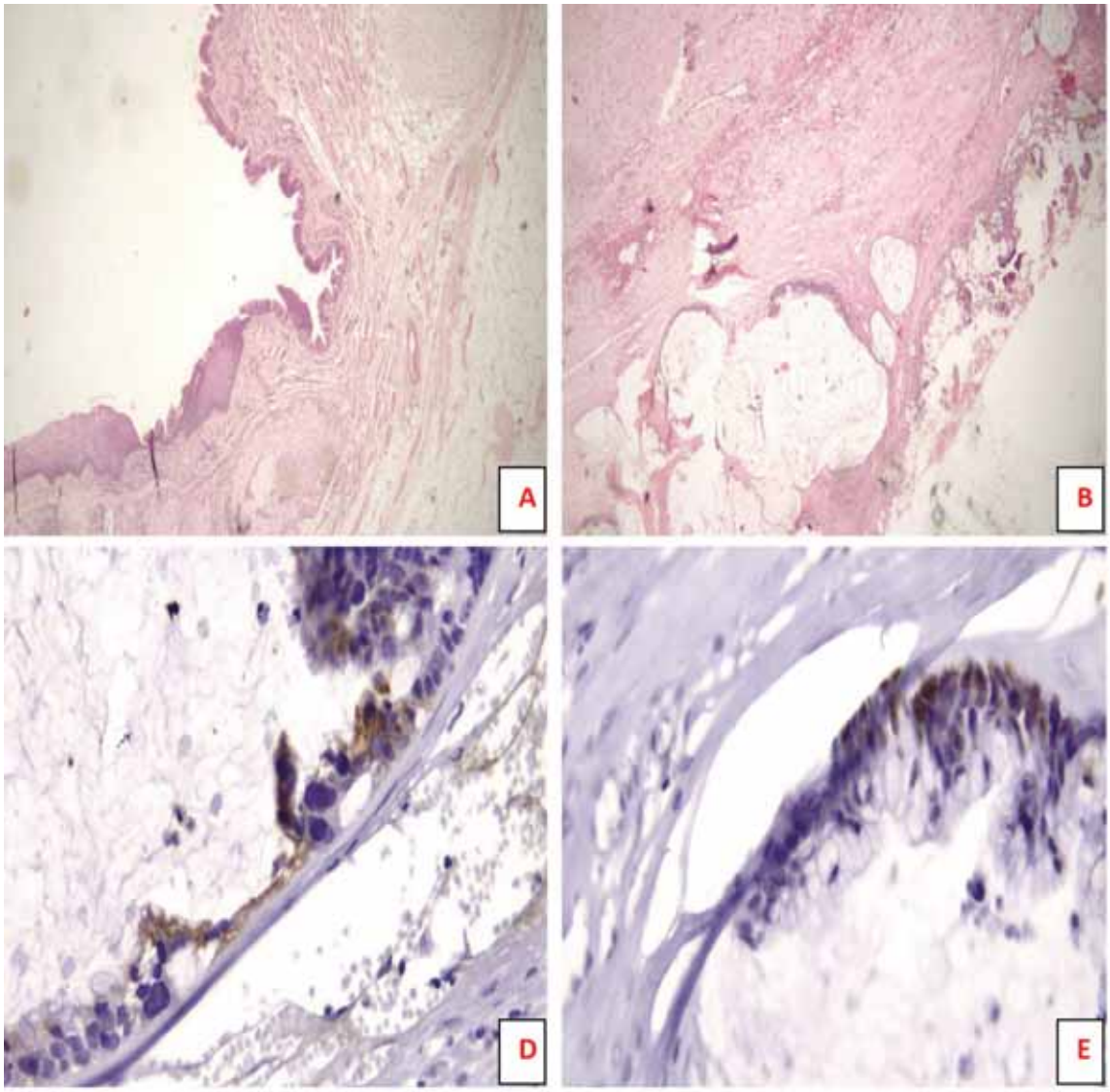

Figure 3. The mature teratoma was composed of cysts lined by mature squamous epithelium simulating skin, with adnexa, as well as foci of benign respiratory-type epithelium and cartilage (A, H&E, 4×). Associated foci of invasive mucinous adenocarcinoma (B, H&E, 4×) showed neoplastic cells floating in pools of extracellular mucin (C, H&E, 40×). Immunohistochemical expression for CK20 (D, H&E, 40×) and CDX2 were detected in tumor cells (E, H&E, 40×).

course of the disease and the unrivaled site of origin by imaging, immune stain, and markers, the old pathology of presacral teratoma was retrieved and re-examined. Surprisingly, a small focus of invasive mucinous carcinoma (intestinal type) was detected with IHC for CK20 and CDX2, revealing focal positive reaction (Figure 3 A-E). The differential diagnosis was ovarian mucinous carcinoma on top of the teratoma or metastatic mucinous carcinoma of GIT origin. However, based on the long history, the presence of incompletely resected teratoma, the lack of response to GIT type chemotherapy, and the known lower CDX2 positivity in ovarian than GIT mucinous tumors, the panel concluded that the diagnosis of primary carcinoma superseded teratoma.

Ethical approval

This case report has been approved by the Institutional Review Board (IRB) of the Mansoura Faculty of Medicine with code R.22.12.1981.

Discussion

The authors herein report the 6th case with this pathology up to our knowledge. The long follow-up sequence of the patient is a point of strength; however, the non-accurate diagnosis and incomplete cytoreduction done at the initial surgery for the patient may be a point of weakness.

Germ cell derivative neoplasms commonly involve sites such as ovaries, followed by testis, anterior mediastinum, retroperitoneum, and sacrococcygeal area. Sacrococcygeal teratoma comprises the most common congenital tumor in the neonatal group with a female predilection; a 4-fold increased incidence is observed in females. However, its incidence in the adult population is relatively uncommon.[6, 7] Malignant transformation occurs in either the embryonic or somatic components, with a low incidence rate of 1%-3% both.[8]

Depending on the tumor extension, the sacrococcygeal tumors are divided into four types. Type I sacrococcygeal tumor has a minimal presacral component with a predominant external component; type II tumors are also predominantly external with definitive intra-abdominal extensions; type III tumors are located mostly in the abdomen or comprise of a dominant pelvic mass extending into the abdomen with a small external component; and type IV are entirely in the presacral region with no external component.[7]

There have been only 2 reported cases of extragonadal teratoma-associated mucinous tumors with pseudomyxoma peritonei, and both had an adverse outcome, suggesting that they may be more aggressive than their gonadal counterparts.[9, 10]

It is difficult to make a diagnosis of malignant transformation of a teratoma preoperatively. However, MRI findings may be helpful in distinguishing malignant transformation from benign neoplasms. Several reports have revealed that an important feature of the malignant transformation of teratoma was the existence of an enhanced solid component,[11] which was also noted in our patient. In addition, a rising CEA was detected in a handsome number of patients,[12] together with our patient, making it a useful aid in diagnosis.

The outcomes for patients with advanced stage mucinous adenocarcinoma superimposed on teratoma are generally poor, and are claimed to be even worse than those of squamous carcinoma arising on teratoma.[13] Surgical resection aimed at complete cytoreduction and avoiding artificial rupture of the tumor should be opted for to prevent recurrence.[12] Unfortunately, this was not the scenario in our patient, given the lack of such knowledge at the time of surgery.

Previously, there were reports of three females[14-16] and two males[7, 17] with mucinous carcinoma arising in a sacrococcygeal teratoma, along with the current report of a female patient. The mean age of the five patients was 49.4 years old, with a median size of 13.5 cm, ranging from 7 to 30 cm in largest diameter. Manifestations vary from pelvic pain, difficult micturition and/or defecation, abdominal swelling, and weight loss. The pathology was mucinous carcinoma in four patients[7, 14, 16, 17] and signet ring carcinoma in one of them.[15] Three received adjuvant chemotherapy;[7, 15, 17] however, no long-term outcome was reported except in the current patient

(seven and a half years since detection of the pelvic teratoma until the last visit).

Conclusion

In conclusion, mucinous carcinomas in sacrococcygeal teratomas are extremely rare. They usually present with non-specific symptoms and are only detected when they have grown to a significant size. Currently, there is no clear guidance regarding adjuvant therapy; however, chemotherapy extrapolating experience in colorectal cancer is commonly used. Long-term outcomes are unknown, although we reported a disseminated metastasis years later. Familiarity of pathologists with this entity and patient counseling with a clear follow-up plan in case of non-straightforward resection of teratomas should be encouraged.

Informed Consent

Informed consent was obtained from the patient.

Conflict of Interest

None declared.

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Calendar of Events

15th European Multidisciplinary Congress on Urological Cancers EMUC 2023
November 02, 2023- November 05, 2023
Marseille, France
Website: https://www.esmo.org/meeting-calendar/emuc-2023

International Conference on Cancer and Clinical Oncology
December 20, 2023- December 21, 2023
Istanbul, Turkey
Website: https://waset.org/cancer-and-clinical-oncology-conference-in-december-2023-in-istanbul

Oncology Nursing Society Annual Congress
April 26, 2023 - April 30, 2023
San Antonio, Texas
Website: https://congress.ons.org/

ASCO 2024 — Gastrointestinal Cancers Symposium 2024
January 18, 2024- January 20, 2024
San Francisco, United States
Website: https://meetings.asco.org/gi/dates-know

ESMO Targeted Anticancer Therapies Congress 2024
February 26, 2024- February 28, 2024
Paris, France
Website: https://www.esmo.org/meeting-calendar/esmo-tat-2024

ESMO Sarcoma and Rare Cancers Congress 2024
March 14, 2024 - March 16, 2024
Lugano, Switzerland
Website: https://www.esmo.org/meeting-calendar/esmo-sarcoma-and-rare-cancers-congress-2024

European Lung Cancer Congress 2024
March 20, 2024 - March 23, 2024
Prague, Czech Republic
Website: https://www.esmo.org/meeting-calendar/european-lung-cancer-congress-2024

Oncology Nursing Society Congress
April 24, 2024 - April 28, 2024
Washington, DC
Website: https://congress.ons.org/

ESMO Gynaecological Cancers Congress 2024
June 20, 2024 - June 24, 2024
Florence, Italy
Website: https://www.esmo.org/meeting-calendar/esmo-gynaecological-cancers-congress-2024

Gastrointestinal Cancers Symposium 2024
September 11, 2024- September 14, 2024
Würzburg, Germany
Website: https://meetings.asco.org/gi/dates-know