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Vol. 49, Issue 3, March 2024

Review Article

Original Articles

Brief Report

Letter to Editor

Mesenteric Panniculitis: An Enigma

Amit Kumar Gupta, Niraj Shrivastava

IJMS
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Review Article

Effect of Fish-Based Diet on Malnourished Children: A Systematic Review

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Abstract

Background: Malnutrition in children is mainly caused by the lack of protein and fat intake which harms their ability to grow and survive. Accurate data on the benefits of fish-based foods on the nutritional status of children is limited. The present systematic review aimed to provide an overview of published articles on the nutritional value of fish-based foods for children.

Methods: A systematic review was performed during 2000-2021 by searching Science Direct, Cochrane Library, PubMed, ProQuest, and Wiley Online Library databases. The full text of selected articles in English was screened based on the inclusion and exclusion criteria. Included articles were all experimental studies (randomized control trial, quasi-randomized trial) or mixed methods studies involving malnourished children. The study was reported under the preferred reporting items for systematic reviews and meta-analyses guidelines. The risk of bias was assessed using the Cochrane tool.

Results: A total of 330,859 articles were screened, out of which eight articles were included in the systematic review. Interventions included fish-based foods and beverages such as wafer bars, Jemawut-tuna cookies, Amizate in chocolate drink, dried fish powder, flaxseed oil supplemented with fish oil capsules, and porridge fortified with fish powder. Primary or secondary outcomes were the determination of zinc level, height growth, erythrocyte n-3 polyunsaturated fatty acid content, safety and acceptability, intestinal integrity, and cognitive development. The results showed that dried fish powder produced the most significant effect on body weight.

Conclusion: The consumption of dried fish powder had positive effects on the recovery of malnourished children.

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Keywords ● Fish flour ● Body weight ● Malnutrition ● Child

What's Known

- Malnutrition is common in children, specially in developing countries. Growth and developmental disorders in children are associated with protein intake below recommended dietary allowance.
- Fish is a good source of nutrients, such as primary macronutrients (protein, fat) and micronutrients (vitamins, minerals). Fish-based diet improves nutritional status during early childhood.

What's New

- Fish-based foods are produced in various forms, not only to preserve nutritional value but also to make fish consumption attractive to children.
- Dried fish powder effectively provides nutrients and improves children's nutritional status. Fish fortified with other ingredients is more effective and the preferred choice to enhance children's health.

Introduction

Adequacy of nutrition is an important issue in every country as it closely relates to national development. It mainly involves young children affecting their cognitive development, education, personality traits, and future productivity.[1-3] Improper feeding practices during infancy and early childhood lead to nutritional deficiencies with the result that they often suffer from infections and intestinal disorders, which in turn impair optimal growth and development.[4] Fish-based foods have been proven to be effective in improving the quality of nutrition in early childhood.[5, 6] Fish contains primary macronutrients (protein and fat) and

micronutrients (vitamins and minerals), and therefore greatly contributes to food security and global nutrition.[3] In developing countries, fish is the main source (75%) of daily animal protein and complementary foods.[7, 8] For example, in Egypt, fish is the main source of animal protein and is popular among the low- and middle-income class, since it is readily available and affordable.[9] Nowadays, fish farming has become a sustainable source of high-quality protein foods.[10, 11]

Inadequate protein intake is closely related to impaired growth and development in children. Physiologically, protein plays an important role in supporting all processes in the human body.[12] Therefore, malnutrition can cause serious health problems and must be addressed immediately. Globally, around 47 million children are stunted, mainly in low- and middle-income countries. In addition, malnutrition and stunted growth are associated with deficiencies in vitamin A, iron, and iodine,[13] which are characterized by blindness, impaired learning, failure to thrive, increased physical weakness, and mortality.[8] Several studies showed that malnutrition is a major risk factor for disease and death in children in Kenya, Uganda, Malawi, Zimbabwe, and Zambia.[14-18] A common factor in these countries is poor economic conditions leading to shortages of nutritious food, such as fish. However, in these countries, aquaculture has great potential to increase access to fish to address food shortages, reduce malnutrition, and improve the nutritional status of children.[11]

Various studies have demonstrated the beneficial effects of fish consumption on body functions as well as the importance of adequate protein intake by children at different stages of their development to adulthood.[19-21] However, the effect of fish consumption on malnutrition has not been fully addressed. To effectively promote community health, the present study aimed to review various published articles on the effects of fish-based diets on malnourished children.

Materials and Methods

A systematic review was conducted on studies published in English between January 2000 to December 2021. A complete search was performed in PubMed, Science Direct, ProQuest, Wiley's online library, and Cochrane Library. The sources were managed using Mendeley reference management software 1.19.8 (Elsevier, Amsterdam, Netherlands) to remove duplications. Based on the specific syntax of various databases, keywords, and phrases (MeSH) including fish flour, malnutrition, and child were used for the search, e.g., Fish flour [Title/Abstract] OR Fish product [Title/Abstract] OR Fish meal [Title/Abstract]) AND Malnutrition [Title/Abstract] OR Malnourishment [Title/Abstract] OR Undernutrition [Title/Abstract].

The inclusion criteria were studies that used fish-based ingredients in any form, assessed body weight, body mass index (BMI), and anthropometric measurement outcomes (both primary and secondary outcomes), and compared the effectiveness of fish-based foods/drinks with other ingredients. The exclusion criteria were studies in adults and pregnant women, food that is not based on fish as the main product, non-English articles, and articles in the form of case reports, reviews, books, and commentaries. PICOS framework (population, intervention, comparison, outcomes, and study) was used to formulate the eligibility criteria (table 1).

The authors (PL, MA) independently reviewed the articles, and disagreements were resolved by another reviewer. The full text of the articles (objectives, methodology, results, and conclusion) was screened based on the inclusion and exclusion criteria. In addition, the reference lists of selected articles were manually reviewed by the first author to extract additional relevant articles. Included articles were all experimental studies (randomized control trial, quasi-randomized trial) or mixed methods studies involving malnourished children.

This study was reported under the Preferred Reporting Items for Systematic Reviews and Meta-analyses (PRISMA) guidelines.[22] The extracted information was evaluated using the GRADE (grading of recommendations, assessment, development, and evaluation) framework, and the associated risk of bias was rated as very low, low, moderate, and high.[23, 24]

Table 1: PICOS (population, intervention, comparison, outcomes, and study) framework used to formulate eligibility criteria for the articles

Items	Statement
Problem	Undernutrition in children
Intervention	Fish-based food
Comparison	Source of food from plants or other animals
Outcome	Anthropometric indices
Study design	Randomized controlled trial, quasi-randomized trial, or mixed methods

Results

A total of 330,859 articles were initially selected, 32,650 through PubMed, 6,243 through Science Direct, 263,707 through ProQuest, 26,611 through Cochrane Library, and 1,648 through Wiley Online Library. Due to various reasons, 330,850 records were excluded, and finally, eight articles were included in the systematic review (figure 1).

Overview of the Included Studies

The studies were conducted in Asia (Cambodia: 2, Indonesia: 1, and India: 1) and Africa (Zambia: 1, Kenya: 1, Gambia: 1, and Malawi: 1). All studies were intervention-based, involving fish as a basic ingredient fortified with other nutrients such as vegetables, fruit, and spices. The participants were children (n=438) aged from three months to seven years who suffered from moderate acute malnutrition (MAM) or severe acute malnutrition (SAM). A summary of the reviewed studies is presented in table 2.

Fish-based Food Intervention

The types of food used for interventions included cylindrical wafer bars with pure fish paste,[25, 32] Jemawut-tuna cookies,[26] Amizate in chocolate drink,[27] dried fish powder (locally called chisense),[28] flaxseed oil containing ready-to-use therapy food (RUTF) with additional fish oil capsules (FFO-RUTF),[29] purified fish oil,[30] and porridge fortified with fish powder.[31] The intervention period varied from 1 month to 1 year. Some studies provided parental nutritional education.[25, 26, 29]

Outcomes

The primary or secondary outcome variables were zinc levels,[26] height growth,[27-32] erythrocyte n-3 polyunsaturated fatty acid (PUFA) content,[29] safety and acceptability,[29] intestinal integrity,[30] and cognitive development.[30]

Effect of Fish-based Nutritional Intervention on Children's Growth

The effect of interventions on malnourished children varied in different countries. While some studies reported a significant effect from the interventions, others reported no statistically significant results. In a study on malnourished children in Cambodia, the intervention involved the use of RUTF in the form of wafer bars for two months and no significant improvements were observed (P>0.05).[25] Another study in Kenya also reported no significant effect of the intervention on the index of linear growth.[29] Similarly, the use of freshwater fish with soy, mung beans, and coconut did not show any improvements in anthropometric indices.[32] On the other hand, some studies reported significant improvements in the parameters of malnutrition. A study in Indonesia used Jemawut-tuna cookies and reported a significant improvement in nutritional status after the intervention (P<0.001).[26]

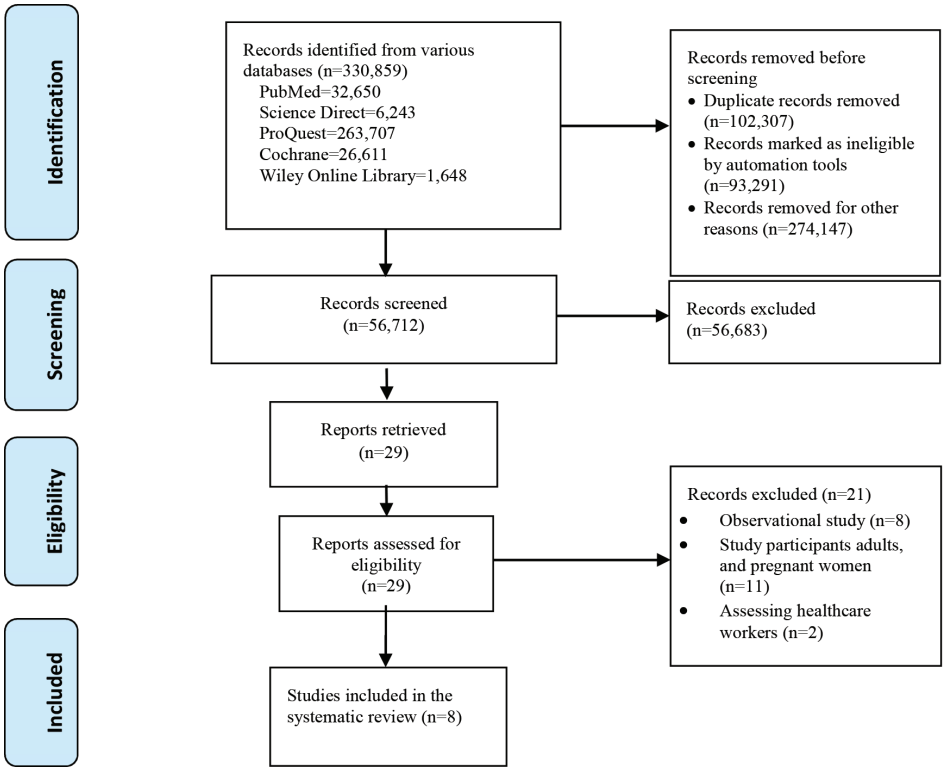

Figure 1: The flow diagram shows the study selection strategies according to PRISMA guidelines.

Table 2: A summary of the studies selected for review

Articles	Objectives	Study design	Participants	Intervention	Evaluations	Outcome	Quality
Sigh et al., 2018, Cambodia[25]	Weight gain	RCT	121 children aged 6-59 months with SAM. Randomly divided into two groups: Control (n=61) treatment with BP-100™ and intervention (n=60) treatment with NumTrey.	The intervention duration was eight consecutive weeks, with follow-up visits every two weeks. Patients aged 6-11 months: Minimum consumption of either 1 wafer with pure fish-based paste (NumTrey) or 1/3 bar (BP-100™). Patients aged 12-23, 24-35, and 36-59 months: A minimum consumption of 1.5 wafers or 1/3 bar, 2 wafers or 2/3 bar, and 3 wafers or 2/3 bar, respectively. Enough to pass the appetite test.	Weight was measured in light clothes (no diapers) to the nearest 100 g. MUAC was measured to the nearest 1 mm (left arm). Bilateral pitting edema was assessed by pressing a finger for 8-10 sec on the foot, hand, and forearm.	There was no statistically significant difference between the two RUTFs (0.02 g/Kg/day, 95% CI: 0.49-0.46). The difference between the two RUTFs was not statistically significant (P>0.05, difference=0.06 g/Kg/day, 95% CI: 0.41-0.54).	High
Ikawati et al., 2020, Indonesia[26]	Weight gain, zinc level	Pre- and post-interven-tion study	48 malnourished children aged 6-24 months. Divided into two groups: Control (n=24) given a biscuit provided by the Ministry of Health and intervention (n=24) given foxtail millet-tuna cookies.	60 days intervention and additional eight sessions of nutritional education.	Weight measurement (not described).	There was a significant difference in nutritional status (weight/age) intake between the intervention and control groups (P<0.001).	Low
Nesse et al., 2014, India[27]	Height, weight, and BMI	RCT	438 malnourished children aged 6-8 years (227 boys and 211 girls) from six government schools	The children were randomized to receive one of the following three interventions for 120 days: (i) a chocolate drink consisting of 60 g of cocoa powder in 120 mL drinking water (placebo), (ii) a chocolate drink containing 3 g/day of Amizate, (iii) a chocolate drink containing 6 g/day of Amizate.	Height, weight, and BMI were measured during each visit.	There was a significant increase in body weight between baseline and four months after the intervention (P<0.05).	Moderate
Chipili et al., 2022, Zambia[28]	Linear growth	RCT	186 infants aged 6-7 months Divided into two groups: Intervention (n=100) and control (n=86).	Infants in the intervention group received 12 g of dried fish (chisense) powder per day, while infants in the control group received 7 g of sorghum powder per day to provide the same energy intake.	Mothers were given a time and day in a month to bring the infant for weight and length measurements.	A significant intervention effect was found between the fish and sorghum groups for WAZ (P<0.05) The addition of fish powder during early complementary feeding improved the infant's linear growth outcome.	High

Articles	Objectives	Study design	Participants	Intervention	Evaluations	Outcome	Quality
Jones et al., 2015, Kenya[29]	Erythrocyte n-3 PUFA content, linear growth, safety, and acceptability	RCT	60 children aged 6 to 50 months with SAM	Standard or flaxseed oil containing RUTF was given to children at a weight-based dose until MUAC was >11.5 cm, weight-for-height/length z-score >-3, or edema had resolved (depending on enrollment criteria) at two consecutive weekly visits. Parents were advised not to give any other food apart from breast milk.	Gas chromatography. Insulin-like growth factor-1 (IGF-1) provided an index of linear growth potential. Anthropometric indices.	There was no difference in IGF-1 between the arms at any time point, but all arms had a highly significant increase in IGF-1 by day 28 compared to baseline, which was sustained to day 84.	High
Van der Merwe et al., 2013, Gambia[30]	Intestinal integrity, growth, and development	RCT	172 infants aged 3-9 months	Supplementation started at three months of age and ended at nine months of age when all outcome measurements were taken apart from cognitive function (assessed at 12 months of age). The intervention group received 2 mL of highly purified fish oil, which supplied 200 mg DHA and 300 mg EPA/d. The control group was given the same volume of olive oil.	Anthropometric measurements. Infant lengths and weights.	Statistically significantly larger MUAC (effect size: 0.31 z-score, 95% CI: 0.06-0.56, P=0.017)	High
Lin et al., 2008, Malawi[31]	Weight gain and height growth during Infancy	Prospective RCT	240 children aged 6-12 months	Mothers of children receiving FP were shown a pre-prepared sample of porridge to demonstrate the appropriate consistency. Each mother then received identical cups and teaspoons and was shown how far to fill the cup to make 1 serving of porridge (70 g). Each mother also received a supply of powdered fish and was shown how many teaspoons (2.5) were to be mixed into the porridge.	Anthropometric indices were calculated using the WHO 2005 standards.	Children who received FS gained more weight than children who received FP from 6-12 months of age, but not from 12-18 months of age (P<0.61).	High
Borg et al., 2020, Cambodia[32]	WAZ, HAZ, WHZ, MUAC	RCT	292 Infants aged 6-11 months	RUSF uses local ingredients, including small freshwater fish, soy, mung beans, and coconut. 40-110 g of RUSF per day, depending on the child's age for six months.	Anthropometric measurements included weight to the nearest 0.1 Kg, recumbent length to the nearest 0.1 cm, and MUAC to the nearest 1 mm.	No statistically significant differences between the groups for any of the anthropometric changes. Mean height increased between 6.4-6.7 cm in all groups. Mean weight increased between 1.20-1.30 Kg in all groups.	High

RCT: Randomized controlled trial; SAM: Severe acute malnutrition; MUAC: Mid-upper arm circumference; RUTF: Ready-to-use therapeutic food; FP: Fish-fortified thickened maize porridge; FS: Fortified spreads; RUSF: Ready-to-use supplementary food; CI: Confidence interval; BMI: Body mass index; WAZ: Weight-for-age z-score; HAZ: Height-for-Age Z-Score; WHZ: Weight-for-height z-score; PUFA: Polyunsaturated fatty acid; DHA: Docosahexaenoic acid; WHO: World Health Organization

However, we rated that study as low-quality with a high risk of bias. Nesse and colleagues used chocolate drinks containing fish protein hydrolysate and reported a significant increase in body weight between baseline and four months post-intervention (P<0.05).[27] In Zambia, the use of chisense significantly increased the body weight of malnourished children (P<0.05).[28] The study in Gambia used purified fish oil and reported a significant increase in mid-upper-arm circumference (MUAC) (P=0.017).[30]

Quality Assessment and Risk of Bias

Quality assessment of the studies, using the GRADE framework, showed that the study by Ikawati and colleagues (Indonesia)[26] was of low quality, and the study by Nesse and colleagues (India)[27] was of moderate quality (table 3). The risk of bias assessment showed that the study by Ikawati and colleagues had a high risk of bias due to the lack of sufficient information related to outcome assessment and lack of randomization accuracy.[26] Three other studies[27, 28, 30] showed one or more areas of some concern (figure 2).

Discussion

Various studies have reported that fish-based foods can address malnutrition in children, specially in low- and middle-income countries. Among all types of fish-based foods, the results showed that dried fish powder produced the most significant effect. A previous study also found that dried fish played an important role in the diet and nutrition of people in Bangladesh.[33] It was reported that fish is a potential source of animal protein that positively affects the growth of infants and children. Its protein content is

Table 3: Summary of the risk of bias assessment for eligible studies

Article	Randomization process	Deviations from the intended intervention	Missing outcome data	Measurement of the outcome	Selection of the reported results	Overall
Sigh et al.[25]	Low	Low	Low	Low	Low	Low
Ikawati et al.[26]	Some concerns	Low	Low	No information	High	High
Nesse et al.[27]	Low	Low	Some concerns	Low	Some concerns	Some concerns
Chipili et al.[28]	Low	Low	Some concerns	Low	Low	Some concerns
Jones et al.[29]	Low	Low	Low	Low	Low	Low
Van der Merwe et al.[30]	Low	Low	Some concerns	Low	Low	Some concerns
Lin et al.[31]	Low	Low	Low	Low	Low	Low
Borg et al.[32]	Low	Low	Low	Low	Low	Low

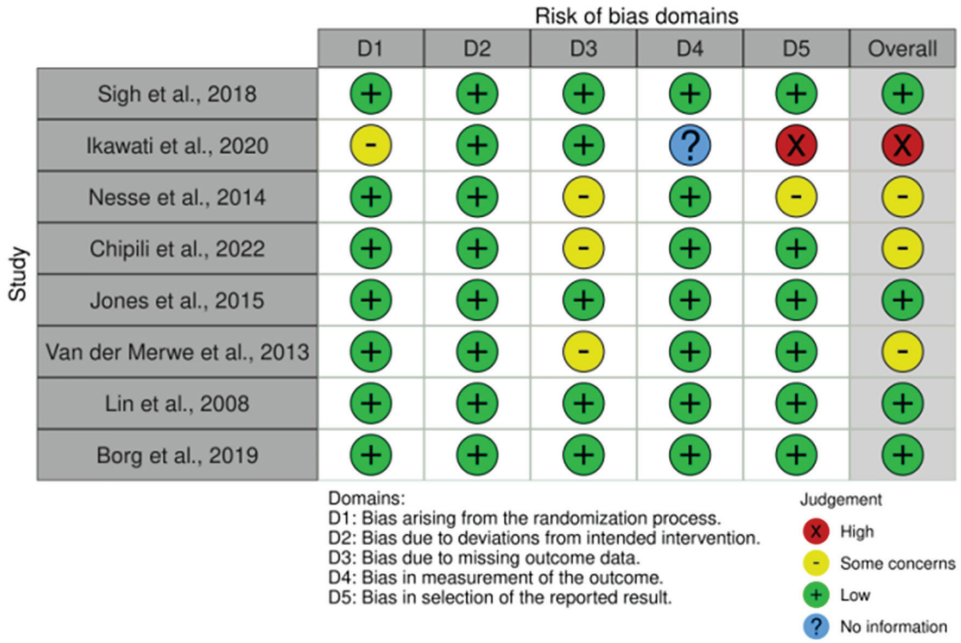

Figure 2: Assessment of the risk of bias of the eligible studies using the five domains in a traffic light plot.

equivalent to beef, chicken, eggs, or liver, while it is cheaper and more affordable for low-income families.[34] Due to n-3 PUFA content, a fish-based diet is important for infants and children to improve their growth. In addition, the proteins and peptides from fish have a high nutritional value and are beneficial for general health. Fish is also a great source of micronutrients, such as vitamins and minerals.[35] While fish is readily accessible in many countries, it can be an expensive and unaffordable food item in some other countries.[36, 37] In the present review study, we mainly focused on the low- to middle-income countries, but did not take into account their financial ability to purchase nutritious food such as fish. Nonetheless, we believe that there are other types of fish-based foods that all people can access and afford.

Of the included articles, two studies reported no statistically significant difference in anthropometric indices (*e.g.*, body weight) after the interventions.[25, 32] The main reasons were stated as low rehabilitation from acute malnutrition in the outpatient treatment of SAM and difference in compliance by patients (*i.e.*, whether the RUTF had been actually consumed, shared with other people, sold, or lost).[25] Borg and colleagues concluded that the reason was due to the low BMI of the mothers during pregnancy.[32] Other studies also confirmed the effect of low BMI during pregnancy on malnutrition in children.[38-41]

Most of the included studies reported a significant effect on anthropometric parameters after intervention with fish-based foods and beverages. Amizate is a fish protein hydrolysate (FPH).[27] FPH is the result of the biological or chemical decomposition of protein derived from fish into its simplest form. In hydrolyzed form, this protein is easily digested and absorbed to enhance the availability of the plasma amino acids.[42-44] FPH from various types of fish has been produced using papain.[45-47]

In other forms of fish-based food, fish oil supplementation has no significant effect on anthropometry, specially the body weight of infants at the age of nine months. However, the positive effect on body weight was observed when they became 12 months of age.[30] This could be due to the slow effect of fish oil on body fat (as indicated by MUAC), which increases skin thickness at the age of 12 months rather than nine months.[45, 48, 49]

The main limitation of the study is a specific focus on published articles from low- to middle-income countries, which undermines the generalizability of our findings. In addition, our findings were negatively affected by the financial capability of the families to purchase nutritious food.

Conclusion

Most of the included studies found that dried fish powder had positive effects on the recovery of malnourished children. In general, fish-based foods had positive effects on malnourished children, and there was no statistically significant difference in its form of production.

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Authors' Contribution

L.B: Study design; P.L, M.A.A: Database search, screening articles, data extraction; L.B, P.L: Risk of bias assessment; M.A.A: Data presentation. All authors have contributed to the writing and revising of the manuscript. They have read and approved the final manuscript and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Conflict of Interest: None declared.

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Original Article

Comparison of High-intensity Laser Therapy with Extracorporeal Shock Wave Therapy in the Treatment of Patients with Plantar Fasciitis: A Double-blind Randomized Clinical Trial

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Abstract

Background: The most common cause of heel pain is plantar fasciitis (PF). Although conservative treatments relieve pain in more than 90% of patients, it may remain painful in some cases. This study aimed to compare High-intensity Laser Therapy (HILT) with Extracorporeal Shock Wave Therapy (ESWT) in patients with PF.

Methods: In this double-blinded randomized clinical trial (conducted in Yazd, Iran, from May 2020 to March 2021), patients were classified into two groups, including the ESWT and HILT, using online randomization. Nine sessions, three times a week for 3 weeks, were the treatment period in both groups. Visual Analogue Score (VAS), Heel Tenderness Index (HTI), and the SF36 questionnaire were compared and analyzed statistically at the beginning and 9 months after treatment.

Results: 38 patients (19 in each group) completed the study. Results showed that pain and patient satisfaction improved significantly 3 months after treatment. The VAS and HTI decreased 3 months after treatment in both groups, which was statistically significant ($P<0.001$). The SF36 score in both groups increased 3 months after treatment, and this increase was statistically significant ($P<0.001$). Although the two modalities were effective based on VAS, HTI, and SF36, a significant statistical difference was observed between them ($P=0.03$, $P=0.006$, $P=0.002$, respectively), and the HILT was more effective.

Conclusion: ESWT and HILT decrease pain and increase patient satisfaction in PF. Besides, both methods are non-invasive and safe. However, there is a significant difference between them, and HILT is more effective.

Trial registration number: IRCT20210913052465N1.

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Keywords ● Physical therapy modalities ● Laser therapy ● Fasciitis, plantar ● Conservative treatment ● Patient satisfaction

What's Known

- Shockwave, steroid injection, and surgery effectively reduce pain and inflammation in patients with plantar fasciitis.
- High-intensity laser therapy restores damaged tissues and eliminates painful irritations by stimulating collagen production, increasing blood flow, increasing vascular permeability, and having anti-inflammatory effects.

What's New

- Both extracorporeal shock wave therapy and high-intensity laser therapy are non-invasive, safe, and effective treatment methods for relieving heel pain. There were no significant differences when these two methods were compared. Laser therapy is preferred due to accessibility and less pain and cost.

Introduction

Repetitive microscopic ruptures in plantar fascia in the medial tubercle of calcaneus result in plantar fasciitis (PF).[1] PF causes chronic pain in the adult population[2, 3] and accounts for nearly

11%-15% of all foot symptoms.[4, 5] Although the suffix "itis" expresses an inflammatory mode, there is increasing evidence that shows this disorder is associated with degenerative changes, so better to be called as "fasciopathy" or fasciosis.[6]

The sex ratio of its occurrence is equal.[3] It is often seen in military personnel, sedentary persons, and athletes.[7] Work activities that need a long-standing position, poor biomechanics of the foot, inappropriate ankle dorsiflexion or excessive foot pronation, higher body mass index (BMI), and weakness of muscles are some of the risk factors.[8-11] Calcaneal spurs may be seen in 50% of radiographies, but the diagnosis is clinical and by ruling out other conditions.[12]

There are many treatment options to relieve symptoms, such as modification of daily or job activities, weight reduction, plantar fascia stretch, physiotherapy, ice massage, non-steroidal anti-inflammatory drugs in combination with other treatment modalities, such as shock wave therapy, local steroidal injection, and surgery.[13, 14]

Pain in almost 10% of PF patients cannot be relieved with conservative treatment.[4] Local corticosteroid injection is a low-cost, available, and effective method of treatment.[15] Extracorporeal shockwave therapy (ESWT), as a recent treatment modality, is a kind of short-duration pulse sound wave with a high-pressure amplitude that may cause an analgesic effect by destroying unmyelinated sensory fibers.[16] High-intensity laser therapy (HILT) is another recent method that can improve pain scores in affected patients.[17]

Clinical studies concluded that low-level laser therapy (LLLT) is an effective and promising treatment for chronic PF.[18, 19] In 2019, Wang and colleagues reviewed six studies in a meta-analysis and concluded that LLLT significantly decreases heel pain in patients with PF, and the efficacy of this treatment lasts for 3 months.[20]

Moreover, the effectiveness of different laser therapies in PF treatment has been evaluated in a clinical trial, and it is concluded that both HILT and LLLT improve the level of pain, function, and quality of life in people with PF. However, HILT has a more significant therapeutic effect than LLLT in PF.[17]

Yesil and colleagues conducted a placebo-controlled study to evaluate the efficacy of HILT on pain, foot function, quality of life, and plantar pressure in patients with plantar heel pain. The results of their study indicated improvement in all parameters in both groups except dynamic pedographic measurement. Additionally, results showed no superiority of HILT over the placebo.[21]

Thus, it can be concluded that there is not enough data on using these new modalities and their adverse effects. This study aimed to compare HILT with ESWT in treating patients with PF.

Patients and Methods

In this double-blinded randomized clinical trial, all patients who referred to the Physical Medicine and Rehabilitation Clinic of Sadoughi Hospital, Yazd, Iran, and diagnosed with PF (tenderness in the medial tubercle of calcaneus and heel pain in the first few steps in the morning, which gets worse with increased activity), from May 2020 to March 2021, were included. They were enrolled in this study if they met the inclusion criteria and gave their consent.

Inclusion criteria were age from 18 to 55 years old, diagnosis of PF confirmed by physical medicine and rehabilitation specialist and orthopedic surgeon, no response to conservative treatments such as ice, soft tissue massage, stretching, usage of insoles, and non-steroidal anti-inflammatory (NSAID) drugs after 6 weeks.

Exclusion criteria included having history of foot surgery in the last 6 months, history of corticosteroid injection in the last 6 months, history of surgery for lumbar disc herniation, diagnosis of rheumatologic disease (rheumatoid arthritis, diffuse idiopathic skeletal hyperostosis (DISH), systemic lupus erythematosus (SLE), gout, Sjogren disease, enthesopathy, and so on), history of heel pain due to trauma in the last 3 months, wounds, infections, and tumors in the treatment area, HILT or ESWT contraindications, no desire to participate in the study, use of any medications that interfere with the healing process or affect the pain, such as glucocorticoids and NSAID drugs.

In this research, considering the confidence level of 95% and the test power of 80% and according to the results of previous similar articles were followed,[22, 23] the standard deviation of the pain level is S=1.05. To achieve a significant difference of at least 0.95 in the average pain level in the intervention groups, 19 people were needed in each group. Considering a 25% drop, the number of 25 people for each group was required, using the below formula.

$$h = \frac{\left(Z\frac{\alpha}{2} + Z\beta\right)^2 \times 2S^2}{(\bar{x}_1 - \bar{x}_2)^2}$$

The study was approved by the Ethics Committee of Shahid Sadoughi University of Medical Sciences (IR.SSU.MEDICINE.REC.1399.017) and was also registered in the Iranian Registry of Clinical Trials (IRCT20210913052465N1). This study was

conducted in accordance with the principles of the Declaration of Helsinki and Iran's ethical codes of research, and before participating in the study, the objectives of the research, the entire process, and its benefits were explained to the participants. Additionally, written informed consent was obtained.

All patients who participated in this study were randomized to HILT or ESWT groups. A random sequence was created using online randomization on www.random.org/integres https://www.random.org/integers/, with a 1:1 allocation using random block sizes of four. Then, the participants were placed in two groups based on their envelopes and codes by the physical medicine and rehabilitation residents. Both the participants and the researcher were unaware of the type of intervention, and the intervention was performed on them by a physical medicine and rehabilitation resident.

Group A received stretching exercises, insoles (if needed), and ESWT, and Group B received stretching exercises, insoles (if needed), and HILT. Medication modalities and adverse effects were described for all patients in the treatment process. Since these services are not covered by insurance, the used equipment was donated, and HILT and ESWT were performed by residents. Therefore, free services were provided to avoid imposing costs on patients. Exercises included towel stretching, plantar fascia stretching, standing calf stretching, and towel pickup, performed three times a day for 2 weeks.

ESWT therapy was done using a Master plus MP100 Shock wave device (STORZ MEDICAL, Switzerland) in the low energy mode.

The first step was done by an R15 transmitter, Bar 2-3, Pulse 3000, and 12 MHZ frequency. The second was done using a D20-S transmitter, Bar 1.8-3, Pulse 3000, and 15 MHZ frequency.

HILT was performed using an Nd: YAG Laser Source GaIA'S (GIGAA LASER, VELASII-30B, United Kingdom) 980±10 nm device.

The device was applied to the plantar fascia area with a voltage of 30 W, a dosage of 8 J/cm^2, and a spot beam diameter area of 10 cm^2.

In both groups, the patient was in a prone position, and treatment was applied to the plantar fascia. Patients' feet were examined and scanned at first, and an insole was administered if needed (such as in patients with *pes planus* or *pes cavus* or other structural disorders in the feet).

In both groups, the treatment period was nine sessions, three times a week, for 3 weeks. Patients were asked to complete the SF-36 questionnaire before the start of treatment and 3 months after the end of treatment. The 36-question quality of life questionnaire (SF-36) has 36 questions and consists of eight subscales including physical function and role disturbance due to physical health. Pain and general health are categorized as physical health and role disturbance due to emotional health, energy/fatigue, emotional well-being, and social functioning are categorized as physical and mental health. Each item is scored from 0 to 100. Scores that are closer to 100, represent a higher quality of life.

The validity and reliability of the Persian version of this questionnaire have been investigated and confirmed in the Montazeri and colleagues' study. They evaluated the reliability of this scale with "internal consistency" and their results showed that except for the Vitality scale (α=0.65), other scales have minimum standard reliability coefficients (0.77 to 0.9).[24] In the current study, nine experts evaluated and confirmed the validity of this scale, with the following results: CVI=0.87 and CVR=0.90.

Visual Analogue Scale (VAS) and Heel Tenderness Index (HTI) were evaluated at the start of treatment and after three months. VAS is one of the scales used for pain rating and is scaled from 0 to 10, where 0 means no pain, and 10 is the most severe pain the patient has experienced. Depending on the amount of pain in the last 48 hours, the person marks it. (0-1: no pain, 2-3: mild pain, 4-5: severe pain, 6-7: very bad pain, 8-9: maximum pain, 10 unbearable pain).[25] HTI is scaled between 0 and 3 when touching the heel (0: no pain, 1: only causes pain, 2: pain with the whine, 3: pain with the whine and withdrawal).

Statistical Analysis

Data analysis was done using SPSS version 22.0 (SPSS, Chicago, IL). The primary characteristics of the patients were reported for quantitative variables as (mean±SD or median [first quartile-third quartile] and for qualitative variables as frequency (percentage). Data distribution was checked using the Shapiro-Wilk test. Paired *t* test was used for the paired comparison of VAS, HTI, SF36, and physical and mental health scores at different times in each group. To compare the effectiveness of two different modalities on pain, an analysis of covariance was used. The homogeneity of variable variances was confirmed using Leven's Test (P>0.05). The significance level of the test was considered to be 5%.

Results

Patient Characteristics

Fifty patients with PF participated in this

randomized clinical trial (RCT). 25 patients received ESWT, and 25 others received HILT. Three patients from the HILT group and three patients from the ESWT group were excluded from the study due to discontinued treatment sessions. Additionally, one patient from the ESWT group was excluded from the study due to bruising. In the continuation of the study process, two patients from the ESWT group and one patient from the HILT group were excluded from the study due to corticosteroid injection, and two patients were lost to follow-up. Finally, 19 people in each group successfully completed the RCT. The flowchart of the study is presented in figure 1.

The results of the present study showed that the majority of participants (71.1%) were men. There is no significant difference between the average age (P=0.77), BMI (P=0.63), and sex (P=0.57) in the ESWT and HILT groups. The frequency distribution of gender was the same in the two treatment groups. Demographic data and measurement results of the patients are presented in table 1.

According to table 2, a one-way ANCOVA was used to compare the effectiveness of two modalities based on VAS, HTI, SF36, and mental and physical health scores. The homogeneity of the variable variances was confirmed using Leven's Test (P>0.05). The results of the test by adjusting the effect of before-treatment scores showed that there was a significant difference in the mean of VAS between these two groups, so that the patients of the HILT group reported less pain (P=0.03). In addition, the HTI mean scores in the ESWT group were significantly less than in the HILT group (P=0.006). Moreover, SF36 and physical health and mental health scores were significantly higher in the HILT group than in the ESWT group (P=0.002, P<0.001, and P=0.008, respectively).

Furthermore, the results of the paired *t* test showed that the decrease of VAS and HTI and the increase of SF 36, physical health, and mental health scores, three months after the treatment compared to before the treatment in each group, was statistically significant.

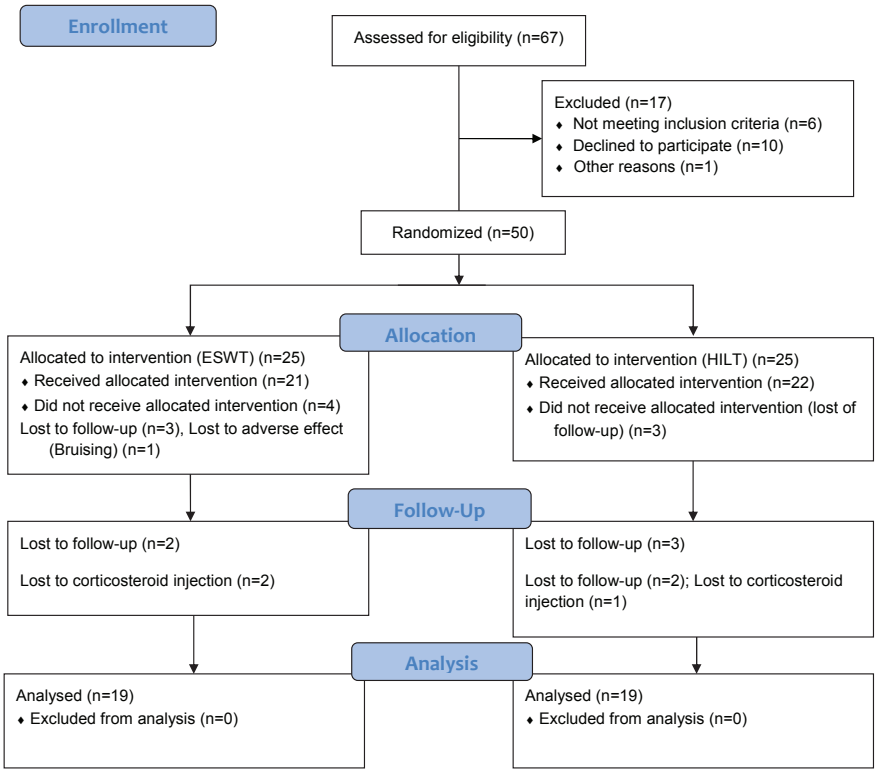

Figure 1: This figure represents the CONSORT flow diagram of the study.

Table 1: Patient demographic information

Variables		Total	ESWT	Laser	P value
Sex	Male	27(71.1)	16 (84.2%)	11 (57.9%)	0.57
	Female	11(28.9)	3 (15.8%)	8 (42.1 %)	
Age		44.65±8.20	45.05±6.85	44.26±9.53	0.77
BMI		27.2±3.51	26.92±3.11	27.47±3.94	0.63

Independent samples *t* test was used. P<0.05 was considered significant. ESWT: Extracorporeal shock wave therapy; BMI: Body mass index

Table 2: Comparison of the HTI, VAS, SF36, and physical and mental health scores within and between the two groups

Variables		ESWT Group Mean±SD	HILT Group Mean±SD	P value Between two Groups
VAS Scores	Before Treatment	7.68±1.53	7.36±1.77	0.03**
	After 3 months	1.68±1.63	1.67±0.99	
In each group at different intervals	P value	<0.001*	<0.001*	
HTI Scores	Before Treatment	2.68±0.95	2.84±0.83	0.006**
	After 3 months	0.42±0.50	0.53±0.69	
In each group at different intervals	P value	<0.001*	<0.001*	
SF36 Scores	Before Treatment	51.42±20.63	59.36±20.51	0.002**
	After 3 months	66.05±14.49	71.52±14.25	
In each group at different intervals	P value	<0.001*	<0.001*	
Physical Health Scores	Before Treatment	48.73±20.70	57.63±18.84	<0.001**
	After 3 months	64.21±14.17	69.58±12.83	
In each group at different intervals	P value	<0.001*	<0.001*	
Mental Health Scores	Before Treatment	56.42±21.91	61.16±22.32	0.008**
	After 3 months	66.79±17.87	71.42±16.69	
In each group at different intervals	P value	<0.001*	<0.001*	

*Paired *t* test was used for comparing variable scores in each group at different intervals, **One-way ANCOVA and Leven's Test were used for comparing variable scores between the two groups. P<0.05 was considered significant. SF36: 36-item short form survey; HTI: Heel tenderness index; VAS: Visual analogue scale; ESWT: Extracorporeal shock wave therapy; HILT: High-intensity laser therapy

Discussion

In this randomized study, we compared two different PF therapies. After the intervention, the score of pain considering the VAS decreased after 3 months in both ESWT and HILT groups, which was statistically significant. Comparing these modalities showed that HILT is more effective.

Similarly, both treatment groups showed a significant decline in HTI scores 3 months after treatment. However, the difference between the ESWT and HILT groups was significant, and the ESWT was more effective.

SF36 and physical and mental health scores improved significantly 3 months after treatment in both ESWT and HILT groups. However, the difference between these two groups was statistically significant, and the HILT was more effective.

Our results were consistent with previous similar studies, which suggest both ESWT and HILT modalities have benefits in reducing pain caused by PF. Similar to our results, a significant improvement in VAS scores was seen at 12 and 24 weeks following ESWT, in the studies by Gollwitzer and colleagues[26] and Dastgir,[27] respectively. VAS score decreased and heel pain improved by 60% in a study by Aqil and others,[28] who compared ESWT with a placebo.

Two other research teams, Kudo and colleagues[29] and Malay and others,[30] also found statistically better outcomes in patients treated with ESWT than those treated with a placebo, whereas Buchbinder and others[10] and Haake and colleagues[31] did not find a significant difference between ESWT and placebo.

Moreover, ESWT was introduced as a safe and effective method for treating chronic pain,[28] which is similar to our findings. Cosentino and colleagues evaluated the ESWT method in patients with heel pain and found a statistically meaningful decrease in VAS scores. They claimed that ESWT is effective in relieving pain, inflammation, and edema.[32] Again in another similar study, ESWT was recommended as a non-invasive method in the treatment approach before surgery.[33]

In contrast to our study, Rompe and colleagues treated their patients with stretching or low-energy shock wave therapy and reported a better mean change in foot function index cumulative score and more patient satisfaction in the stretching group.[34] Again in a study by Speed and others, shock wave therapy was ineffective compared to a placebo in a group of adults with PF.[35]

The differences between studies on ESWT efficacy in patients with PF may be multifactorial, including different study populations, using various machines or methods, and variations in treatment parameters such as intensity, focus target, and focal energy of shock waves. Additionally, using different outcome measuring

scales may cause bias in the comparison between various studies. There is no consensus on the best dosage and other treatment parameters of ESWT for PF.

Guimaraes and others conducted a systematic review and compared LLLT to ESWT in the treatment of patients with PF. They concluded that LLLT may improve pain in the short term and can be considered a component of PF patients' care. However, this superiority is not clear compared to ESWT.[36]

Koz and colleagues compared the efficacies of ESWT and LLLT in the treatment of PF patients. The results of this study indicated a significant reduction in pain, and an improvement in functional status, and daily life activities for both treatments. It also showed that the use of LLLT is more effective than ESWT in reducing pain.[37] Tkocz and others investigated the effect of HILT versus a placebo-controlled group on the management of painful heel spurs and PF. They found no statistically significant differences between the two groups. They concluded that HILT does not appear to be more effective in managing pain in patients with heel spurs and PF than standard conservative methods of physical therapy.[38]

According to Yesil and others, no difference between HILT and placebo was found in terms of pain, quality of life, and functionality in the management of painful calcaneal spur. However, they found a significant difference in favor of HILT in dynamic pedographic measurements.[21]

A systematic review by Ezzati and others suggested that it cannot yet be concluded that HILT is an effective non-invasive treatment for managing musculoskeletal pain. However, this treatment may have benefits in some conditions. As a result, to determine the effect of HILT on pain reduction, long-term and randomized controlled trials with appropriate methodological designs are needed.[39]

Dovile and colleagues conducted a randomized participant-blind controlled trial study and compared the HILT with LLLT in the management of PF. Their results showed that there is no statistically significant difference between the two groups according to VAS, pressure algometry, and sonography measurements. Although the reduction of plantar fascia thickness 3 weeks from baseline was statistically significant in the HILT group, and this reduction in HILT may be faster than LLLT, there was no statistically significant difference between the two groups.[40]

Ordahan and colleagues investigated the effect of HILT versus LLLT in the management of PF. They concluded that all evaluated parameters, including VAS and HTI scores, and FAOS showed significant improvements after 3 weeks of treatment in both groups. The improvement of all parameters in the HILT group was more significant than in the LLLT group.[17]

Although many studies show the efficacy of low-intensity laser therapy (LILT) in PF, few investigations have examined the effect of HILT in PF treatment. LILT and HILT are the two most commonly used laser methods in musculoskeletal disorders.

One of the limitations of the present study is the lack of long-term follow-up results. However, we believe that our study is valuable because it is one of the first studies in the literature to compare ESWT and HILT in patients with PF.

Another limitation of our study was the minimum number of treatment sessions due to specific conditions caused by the COVID-19 pandemic. To strengthen the findings of the research, more studies are suggested to be done with further treatment sessions, larger sample sizes, and a higher number of investigation groups, including control groups with placebo, to make a better comparison of the treatment methods.

Conclusion

As seen in our study, both ESWT and HILT treatment methods are non-invasive, safe, and effective therapies in relieving heel pain based on different pain scales and patient satisfaction questionnaires. However, HILT is preferred because it is more effective in the improvement of pain (based on VAS) and quality of life and is also more accessible with less pain and cost.

Authors' Contribution

M.ZB: Study conception, study design, data acquisition, data analysis, and interpretation, drafting the manuscript; MR.VN: Study conception, study design, revising the manuscript critically; A. Kh A: Study conception, study design, data acquisition, revising the manuscript critically; All authors have read and approved the final manuscript and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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Original Article

Evaluation of Some Prognostic Biomarkers in Human Papillomavirus-Related Multiphenotypic Sinonasal Carcinoma

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What's Known

- Human papillomavirus (HPV)-related multiphenotypic sinonasal carcinoma (HMSC) is a novel form of sinonasal carcinoma associated with high-risk HPV.
- HMSC is a rare distinct tumor with high-risk local recurrence, unknown clinicopathologic spectrum, and prognosis, and is often misdiagnosed as adenoid cystic carcinoma of the salivary gland or sinonasal squamous cell carcinoma.

What's New

- Forty patients with morphological characteristics of HMSC were evaluated for high-risk HPV, the absence of adenoid cystic carcinoma-related proteins, and the presence of squamous and myoepithelial proliferation.
- The expression of some biomarkers was associated with aggressive malignant behavior, poor survival, and poor prognosis.

Abstract

Background: Human papillomavirus (HPV)-related multi phenotypic sinonasal carcinoma (HMSC) is a recently described tumor subtype with an unknown prognosis, often misdiagnosed with other sinonasal carcinomas, and associated with high-risk HPV (HR-HPV). The present study aimed to evaluate the expression of vascular endothelial growth factor (VEGF), Bcl-2-associated X protein (BAX), epidermal growth factor receptors (EGFR), ProEx™C, and human telomerase reverse transcriptase (hTERT) and assess their association with survival and clinicopathological characteristics.

Methods: Between 2017 and 2022, 40 HMSC patients underwent surgical resection at the School of Medicine, Zagazig University Hospitals (Zagazig, Egypt). Tissue samples were examined for the presence of HR-HPV; absence of myeloblastosis (MYB), MYB proto-oncogene like 1 (MYBL1), and nuclear factor I/B (NFIB) fusions and the presence of myoepithelial proteins (calponin, S100, SMA), squamous differentiation markers (p63, p40, calponin), VEGF, BAX, ProEx™C, and hTERT by immunohistochemistry. All patients were followed up for about 54 months until death or the last known survival data. Data were analyzed using the Chi square test and Kaplan-Meier method.

Results: The expression of VEGF, hTERT, and ProEx™C was significantly associated with age, advanced tumor stages, lymph node metastasis, tumor size, mortality, relapse, poor disease-free survival (DFS), and overall survival (OS) ($P<0.001$). BAX expression was significantly associated with tumor size, age, poor DFS, and relapse ($P=0.01$, $P<0.001$, $P=0.035$, and $P=0.002$, respectively).

Conclusion: HMSC is strongly associated with HR-HPV. The expression of VEGF, EGFR, BAX, hTERT, and ProEx™C is associated with aggressive malignant behavior, poor survival, and poor prognosis, making them novel prognostic biomarkers for targeted therapeutics in HMSC.

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Introduction

Sinonasal cancer (SNC) accounts for approximately 3.6% of all head and neck malignancies and less than 0.2% of all cancers.

The annual incidence of SNC is 0.556 cases per 100,000 people.[1] Between 2016 and 2021, orofacial malignancies accounted for 3.54% of all head and neck cancers in Egypt.[2] Histological subtypes of SNC include adenoid cystic carcinoma (ACC), squamous cell carcinoma (SCC), and some other less common subtypes. These malignancies originate from seromucous glands and surface epithelium.[3] The exact cause of SNC remains unclear. However, smoking is considered a significant risk factor in most head and neck cancers. It was reported that intestinal-type sinonasal adenocarcinoma is related to occupational exposure to wood dust.[4]

Human papillomavirus (HPV) is widely accepted as the cause of 20-25% of all head and neck cancers. Most HPV-related head and neck tumors occur in the oropharynx. However, SNC accounts for 20-25% of these tumors. High-risk HPV (HR-HPV) infection is strongly associated with HPV-related multiphenotypic sinonasal carcinoma (HMSC), especially the HPV-33 strain.[5] HMSC is usually found as tissue fragments focally lined by respiratory epithelium with occasional squamous metaplasia. Histologically, HMSC is divided by fibrous hyalinizing bands into compartments with two distinct patterns, namely cribriform and solid. The cribriform pattern consists of cylindromatous microcystic spaces with basophilic mucoid material surrounded by basaloid tumor cells. In contrast, the solid pattern shows compact tumor cells with a minimum amount of eosinophilic cytoplasm and multiple atypical cells with nuclear pleomorphism and vesicular nuclei, with different areas showing high mitotic activity (50-55 mitoses per 10 high-power fields) and confluent necrosis.[5] HMSC cribriform pattern is morphologically similar to ACC but lacks translocation between myeloblastosis (MYB), MYB proto-oncogene like 1 (MYBL1), and nuclear factor I/B (NFIB). On the other hand, the solid pattern mimics SCC but differs by the presence of myoepithelial differentiation.[6] The prevalence of HMSC is still unclear. However, it appears to be less aggressive than sinonasal SCC but has a higher risk of local recurrence in up to 36% of all cases.[5] HMSC cribriform patterns are often misdiagnosed as ACC, and solid patterns as SCC. HPV encodes two late genes (L1 and L2) and six earlier genes (E1-E7). E5, E6, and E7 are the main oncogenes involved in cell proliferation and aid viral replication. These viral oncogenes can promote tumorigenesis by activating different molecular signaling pathways.[7]

To date, due to its rarity, the molecular biology of HMSC has not been well-studied. It is known that the expression of vascular endothelial growth factor (VEGF), ProEx™C, Bcl-2-associated X protein (BAX), epidermal growth factor receptor (EGFR), and human telomerase reverse transcriptase (hTERT) is associated with poor prognosis of ACC of the salivary gland. However, their expressions in HMSC have not been previously evaluated. Therefore, the present study aimed to evaluate the expression of VEGF, BAX, EGFR, hTERT, and ProEx™C in patients with HMSC and assess their association with survival and clinicopathological characteristics.

Patients and Methods

A total of 40 patients with HMSC who underwent surgical resection with adjuvant radiation therapy (if needed) or definitive concurrent chemoradiation were enrolled in the study. Between 2017 and 2022, these patients were treated in various departments of the Faculty of Medicine, Zagazig University Hospital (Zagazig, Egypt). Follow-up was scheduled every three months in the first two years and every six months in subsequent years. All patients were followed up for about 54 months (range: 20-60) until death or last known survival data.

The patients were classified according to the TNM staging system (tumor size, extent of spread to the lymph nodes, and presence of metastasis) by the American Joint Committee on Cancer (AJCC) for sinonasal neoplasms.[8] Only patients who tested positive for HR-HPV; negative for MYBL1, MYB, and NFIB fusions; and positive for myoepithelial (calponin, SMA, S100) and squamous (p40, p63) differentiation markers were included in the study. The tissue samples were evaluated for the following:

- The presence of HR-HPV (a high-risk cocktail) using polymerase chain reaction (PCR).
- The absence of MYBL1, MYB, and NFIB fusions using fluorescence *in situ* hybridization (FISH) to rule out ACC.
- The presence of myoepithelial (calponin, SMA, S100) and squamous (p40, p63) differentiation markers for immunohistochemical identification of cancer.
- The expression of VEGF, ProEx™C, BAX, EGFR, and hTERT using immunohistochemistry technique and their association with clinicopathological and prognostic parameters of all patients.

The study was carried out in accordance with the ethical principles proposed by the World Medical Association for Human Studies,[8] and was approved by the Ethics Committee of Zagazig University (number: ZU-IRB#9902). Written informed consent was obtained from all patients.

HPV Genotyping Assay

Quantitative HPV-specific PCR was performed for HPV genotyping of HMSC tissue samples. DNA was extracted from 5 µm thick slides containing paraffin-embedded tumor tissues. The tissues were macrodissected from the slides, deparaffinized with xylene, and digested with 50 g/mL proteinase K (Boehringer Mannheim, Mannheim, Germany) in a solution containing 1% sodium dodecyl sulfate at 48 °C for two days. In accordance with the manufacturer's recommendations, the DNA was extracted using ultrapure chloroform:phenol: isoamyl alcohol reagent (Invitrogen, Carlsbad, CA, USA).[9]

The L1 area of the HPV genome was amplified by consensus primers Gp5+-Gp6+ and Gp5-Gp6 using 30 µl PCR solution consisting of ammonium sulfate (16.6 mmol), tris Trizma™ crystals (67.0 mmol at pH 8.8), magnesium chloride (6.70 mmol), ethyl mercaptan (10.0 mmol), dimethyl sulfoxide (0.1 %), DMSO (3.3%), each primer (20 pmol), and platinum Taq (0.5 U).[10] The procedure for the rapid PCR in a Veriti thermal cycler was 40 cycles at 95 °C for 30 seconds, 44 °C for 60 seconds, and 72 °C for 90 seconds. Type-specific primers were used for the E6 and E7 regions of HPV types 11, 16, 18, 31, 33, 35, and 56.[11] The amplification cycle was reduced to 35 cycles, and the annealing temperature for HPV-33 and HPV-35 primers was set to 57 °C for 30 seconds.

Fluorescence in Situ Hybridization

Break-apart FISH assay for MYB, NFIB, and MYBL1 (all from Empire Genomics, Buffalo, NY, USA) was performed.[10] Tumour cells were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) II (ZytoVision GmbH, Bremerhaven, Germany) after hybridization.[12]

Immunohistochemistry

The tissue samples were deparaffinized for 15 min in a 56 °C oven, sectioned at a 3-5 µm thickness, and fixed on positively charged slides, and then placed in xylene for 30 min. The slides were hydrated in descending alcohol series (concentrations 95%, 85%, and 75% alcohol) for five min. The samples were then washed with phosphate-buffered saline (PBS) for 5 min.[9, 13] Antigen retrieval was performed by microwaving the samples for 20 min in a ready-to-use Dako target recovery solution (PH 6.0). Using a lint-free tissue (gauze pad), the residual liquid around the sample was carefully removed to keep the reagent within the defined area.[14-18] To inhibit endogenous peroxidase, tissue sections were treated with 3% hydrogen peroxide, incubated for 5 min, and then carefully rinsed with distilled water.

Primary antibodies were VEGF monoclonal antibody (H11), catalog number MA5-13182 in a 1:20 dilution; BAX monoclonal antibody, catalog number MA5-14003 in a 1:50 dilution; and EGFR monoclonal antibody (JH121), catalog number MA5-13070 in 2 µg/mL dilution (all from Invitrogen, Thermo Fisher Scientific, USA). Furthermore, we used ProEx™C (prediluted, clone MCM2 26H6.19, MCM2 27C5.6, TOP2A SWT3D1; 3D Imaging Inc, Burlington, NC, USA); anti-hTERT, anti-telomerase catalytic subunit (RABBIT) antibody 600-401-252S (Rockland Immunochemicals, Inc., Limerick, PA, USA), SMA and S100 (clone HHF35 and 4C4.9, respectively; Ventana Medical Systems, Inc. Tucson, AZ, USA), and calponin (clone CALP; DAKO GmbH, Jena, Germany).

The tissue specimens were washed in PBS and incubated for 15 min at room temperature with biotinylated anti-mouse immunoglobulin.[19-22] Streptavidin-HRP was added to the tissue slides and washed after 15 min. Next, they were treated with diaminobenzidine (DAB) substrate, incubated for five to 10 min, and then gently washed with distilled water. The slides were submerged in Mayer's hematoxylin solution and incubated for 2-5 min, depending on the hematoxylin strength. DPX was used as mounting medium, and the tissue slides were carefully mounted with a coverslip after clearing in three changes of xylene.

Scoring System for Immunohistochemical Staining

VEGF and BAX positivity was detected as cytoplasmic staining, ProEx™C as nuclear, hTERT as cytoplasmic, nuclear, or both; and EGFR as cytoplasmic and membrane staining. The immune response in tissue samples was identified in 10 randomly selected fields by counting the percentage of stained cells in each field. These were then scored as negative staining (score 0), 1-25% stained cells (score 1), 26-50% stained cells (score 2), 51-75% stained cells (score 3), and 76-100% stained cells (score 4). The intensity of staining was scored as negative (score 0), mild (score 1), moderate (score 2), and high (score 3) intensity. The final result was deduced by multiplying the intensity score by the percentage of positive cell fraction.[19] ProEx™C nuclear staining was scored as negative (<5% of the nuclei are stained), weak (5-25% of the nuclei are positive), moderate (25-50% of the nuclei are positive), and strong (>50% of the nuclei are stained). Two pathologists blinded to the clinical data of the patients independently evaluated all slides.

Statistical Analysis

Data were analyzed using GraphPad Prism statistical software, version 7 (GraphPad Prism Software Inc., San Diego, CA, USA). The Chi square test was used to analyze the expression levels and their association with prognostic and clinicopathological parameters. Kaplan-Meier method was used to estimate the overall survival (OS) and disease-free survival (DFS) and the log-rank test to analyze the difference. Univariable and multivariate Cox regression was used to assess the effect of all variables. A two-sided P≤0.05 was considered statistically significant.

Table 1: Demographic and Clinicopathological parameters of the patients

Parameters		Patients (n, %) (N=40)
Age (years)	55.4±14.4* (range: 29-83)	
Sex	Male	16 (40%)
	Female	24 (60%)
Age group (years)	<45	8 (20%)
	≥45	32 (80%)
Primary site	Paranasal sinus	12 (30%)
	Nasal cavity	26 (65%)
	Orbit	2 (5%)
HPV type	HPV-33	34 (85%)
	HPV-35	4 (10%)
	HPV-16	2 (5%)
Tumor size	T1/T2	24 (60%)
	T3/T4	16 (40%)
Lymph node metastasis	N0	26 (65%)
	N1	6 (15%)
	N2	4 (10%)
	N3	4 (10%)
Stage	Early stage (I, II)	26 (65%)
	Advanced stage (III, IV)	14 (35%)
Distant metastasis	M0	39 (97.5%)
	M1	1 (2.5%)
VEGF expression	Low	10 (25%)
	High	30 (75%)
EGFR expression	Negative	11 (27.5%)
	Positive	29 (72.5%)
BAX expression	Negative	8 (20%)
	Positive	32 (80%)
ProEx™C expression	Negative	16 (40%)
	Positive	24 (60%)
hTERT expression	Low	18 (45%)
	High	22 (55%)
Treatment modality	Surgery	16 (40%)
	Sur+rt±cth	20 (50%)
	CCRT	4 (10%)
Relapse	Absent	16 (40%)
	Local recurrence	20 (50%)
	Distant metastasis	2 (5%)
	Died	2 (5%)
Treatment after recurrence	Surgery	8 (20%)
	Sur+rt±cth	4 (10.53%)
	Sur+reirrad±cth	6 (15.79%)
	CCRT	2 (5.26%)
	CTH	2 (5.26%)
	No TTT	16 (42.11%)
Mortality	Alive	26 (68.42%)
	Dead	12 (31.58%)

*Mean±SD (standard deviation); HPV: Human papillomavirus; VEGF: Vascular endothelial growth factor; EGFR: Epidermal growth factor receptor; BAX: Bcl-2-associated X protein; hTERT: Human telomerase reverse transcriptase; Sur+rt±cth: Surgery followed by postoperative irradiation with/without chemotherapy; Sur+reirrad±cth: Surgery followed by postoperative re-irradiation with/without chemotherapy; CCRT: Chemoradiotherapy; CTH: Chemotherapy; TTT: Transpupillary thermotherapy

Table 2: Association of clinicopathological and outcome parameters with the expression of VEGF, BAX, EGFR, ProEx™C, and hTERT in 40 HMSC patients

Parameters			VEGF		P value	BAX		P value	EGFR		P value	ProEx™C		P value	hTERT		P value
			Low (n=10)	High (n=30)		Low (n=8)	High (n=32)		Negative (n=11)	Positive (n=29)		Negative (n=14)	Positive (n=26)		Low (n=18)	High (n=22)	
		Total	n (%)			n (%)			n (%)			n (%)			n (%)		
Sex	Male	16	4 (25%)	12 (75%)	0.649	5 (31.2%)	11 (68.8%)	0.292	4 (25%)	12 (75%)	0.533	10 (62.5%)	6 (37.5%)	0.582	7 (43.8%)	9 (56.2%)	0.578
	Female	24	6 (25%)	18 (75%)		9 (37.5%)	15 (62.5%)		7 (29.2%)	17 (70.8%)		12 (50%)	12 (50%)		11 (45.8%)	13 (54.2%)	
Age group (years)	<45	8	7 (87.5%)	1 (12.5%)	<0.001*	6 (75%)	2 (25%)	<0.001*	8 (100%)	0 (0%)	<0.001*	8 (100%)	0 (0%)	<0.001*	8 (100%)	0 (0%)	<0.001*
	≥45	32	3 (9.4%)	29 (90.6%)		2 (6.2%)	30 (93.8%)		3 (9.4%)	29 (90.6%)		6 (18.8%)	26 (81.2%)		10 (31.2%)	22 (68.8%)	
Primary site	Paranasal sinus	12	6 (50%)	6 (50%)	<0.001*	5 (41.7%)	7 (58.3%)	<0.001*	7 (58.3%)	5 (41.7%)	0.008	9 (75%)	3 (25%)	<0.001*	9 (75%)	3 (25%)	0.039
	Nasal cavity	26	3 (11.5%)	23 (88.5%)		1 (3.8%)	25 (96.2%)		3 (11.5%)	23 (88.5%)		4 (15.4%)	22 (84.6%)		8 (30.8%)	18 (69.2%)	
	Orbit	2	1 (50%)	1 (50%)		2 (100%)	0 (0%)		1 (50%)	1 (50%)		1 (50%)	1 (50%)		1 (50%)	1 (50%)	
Tumor size	T1/T2	24	10 (41.7%)	14 (58.3%)	0.002	8 (33.3%)	16 (66.7%)	0.01	11 (45.8%)	13 (54.2%)	0.001	14 (58.3%)	10 (41.7%)	<0.001*	18 (75%)	6 (25%)	<0.001*
	T3/T4	16	0 (0%)	16 (100%)		0 (0%)	16 (100%)		0 (0%)	16 (100%)		0 (0%)	16 (100%)		0 (0%)	16 (100%)	
Lymph node metastasis	Absent	26	10 (38.5%)	16 (61.5%)	0.006	7 (26.9%)	19 (73.1%)	0.140	11 (42.3%)	15 (57.7%)	0.004	14 (53.8%)	12 (46.2%)	<0.001*	18 (69.2%)	8 (30.8%)	<0.001*
	Present	14	0 (0%)	14 (100%)		1 (20%)	13 (92.9%)		0 (0%)	14 (100%)		0 (0%)	14 (100%)		0 (0%)	14 (100%)	
Distant metastasis	M0	39	10 (25.6%)	29 (74.4%)	0.750	8 (20.5%)	31 (79.5%)	0.800	11 (28.2%)	28 (71.8%)	0.725	14 (35.9%)	25 (64.1%)	0.650	18 (46.2%)	21 (53.8%)	0.55
	M1	1	0 (0%)	1 (100%)		0 (0%)	1 (100%)		0 (0%)	1 (100%)		0 (0%)	1 (100%)		0 (0%)	1 (100%)	
Stage	Early (I/II)	26	10 (38.5%)	16 (61.5%)	0.006	7 (26.9%)	19 (73.1%)	0.140	11 (42.3%)	15 (57.7%)	0.004	14 (53.8%)	12 (46.2%)	<0.001*	18 (69.2%)	8 (30.8%)	<0.001*
	Advance (III/IV)	14	0 (0%)	14 (100%)		1 (7.1%)	13 (92.9%)		0 (0%)	14 (100%)		0 (0%)	14 (100%)		0 (0%)	14 (100%)	
Relapse	Absent	16	9 (56.2%)	7 (43.8%)	<0.001*	5 (31.2%)	11 (68.8%)	0.002	11 (100%)	6 (37.5%)	<0.001*	14 (87.5%)	2 (12.5%)	<0.001*	13 (81.2%)	3 (18.8%)	0.001
	Present	22	0 (0%)	22 (100%)		1 (4.5%)	21 (95.5%)		0%)	22 (100%)		0 (0%)	22 (100%)		4 (18.2%)	18 (81.8%)	

Parameters		Total	VEGF		P value	BAX		P value	EGFR		P value	ProEx™C		P value	hTERT		P value
			Low (n=10)	High (n=30)		Low (n=8)	High (n=32)		Negative (n=11)	Positive (n=29)		Negative (n=14)	Positive (n=26)		Low (n=18)	High (n=22)	
			n (%)			n (%)			n (%)			n (%)			n (%)		
Mortality	Dead	12	0 (0%)	12 (100%)	0.015	2 (16.7%)	10 (83.3%)	0.548	1 (8.3%)	11 (91.7%)	0.185	1 (8.3%)	11 (91.7%)	0.021	1 (8.3%)	11 (91.7%)	0.002
	Alive	28	10 (35.7%)	18 (64.3%)		6 (21.4%)	22 (78.6%)		10 (357%)	18 (643%)		13 (46.4%)	15 (53.6%)		17 (60.7%)	11 (39.3%)	
Disease-free survival (DFS)																	
Mean (months)			58.9	47.364	0.001	56.25	49.2	0.035	59.00	47.016	<0.001*	59.214	45.474	<0.001*	56.667	45.627	<0.001*
Median DFS			NR	48.0		NR	52.0		NR	48.00		NR	44.0		NR	40.0	
5-year DFS			90%	23%		87.5%	32.9%		90.9%	22.1%		92%	13%		77%	15%	
Overall survival (OS)																	
Mean (months)			60.0	56.22	0.113	58.857	56.723	0.262	60.00	56.08	0.08	60.0	55.61	0.021	59.882	54.786	0.002
Median OS			NR	NR		NR	NR		NR	NR		NR	NR		NR	58.0	
5-year OS			88.9%	62.1%		85.7%	64.6%		88.9%	60.8%		91.7%	56.1%		94.1%	47.7%	

NR: Not reached

Results

A total of 40 patients were diagnosed with HMSC and their demographic and clinicopathological characteristics were obtained (table 1). The patients were divided into two age groups, namely <45 years old (n=8) and ≥45 years old (n=32). Based on the TNM staging system, the size of the tumor in 24 (60%) and 16 (40%) patients were in stages T1/T2 and T3/T4, respectively. The nasal cavity was the primary site of the tumor with no lymph node involvement in 26 (65%) patients. Lymph node metastasis N1, N2, and N3 was observed in 6 (15%), 4 (10%), and 4 (10%) patients, respectively. During the initial evaluation, there was no distant metastasis in 39 (97.5%) patients. Of all patients, 26 (65%) had tumor stage I/II, and 14 (35%) had advanced tumor stage III/IV. Treatment modality in 16 (40%) patients was only surgery, 20 (50%) received postoperative irradiation with/without chemotherapy, and 4 (10%) received chemoradiotherapy. There was no relapse in 16 (40%) patients, 20 (50%) had local recurrence, 2 (5%) had distant metastases after treatment, and 2 (5%) patients died. Of the patients with relapse, 8 (20%) underwent surgery, 4 (10.53%) underwent surgery plus irradiation with/without chemotherapy, 6 (15.79%) underwent surgery plus re-irradiation with/without chemotherapy, 2 (5.26%) received chemoradiotherapy, and 2 (5.26%) received chemotherapy.

Immunohistochemical Evaluations

The results of immunohistochemistry tests for the expression of VEGF, BAX, EGFR, ProEx™C, and hTERT in relation to clinicopathological parameters of all patients are presented in table 2.

VEGF: Of the 40 patients, 30 (75%) showed high expression, and 10 (25%) had low expression of VEGF (figures 1A and 1B). A strong association was found between high VEGF expression and tumor size (P=0.002), ≥45 age group (P<0.001), advanced tumor stages III and IV (P=0.006), and lymph node metastasis (P=0.006). Relapse was associated with high positive expression of VEGF compared to patients with negative expression (P<0.001). The five-year DFS in patients with low VEGF expression was 90% compared to those with high VEGF expression (23%) (P=0.001). However, the five-year OS was significantly higher in patients with low VEGF expression (88.9%) than those with high expression (62.1%). Furthermore, there was a significant association between high VEGF expression and mortality (P=0.015).

BAX: Of the 40 patients, 32 (80%) showed high expression, and 8 (20%) had low expression of BAX (figures 1C and 1D). High BAX expression

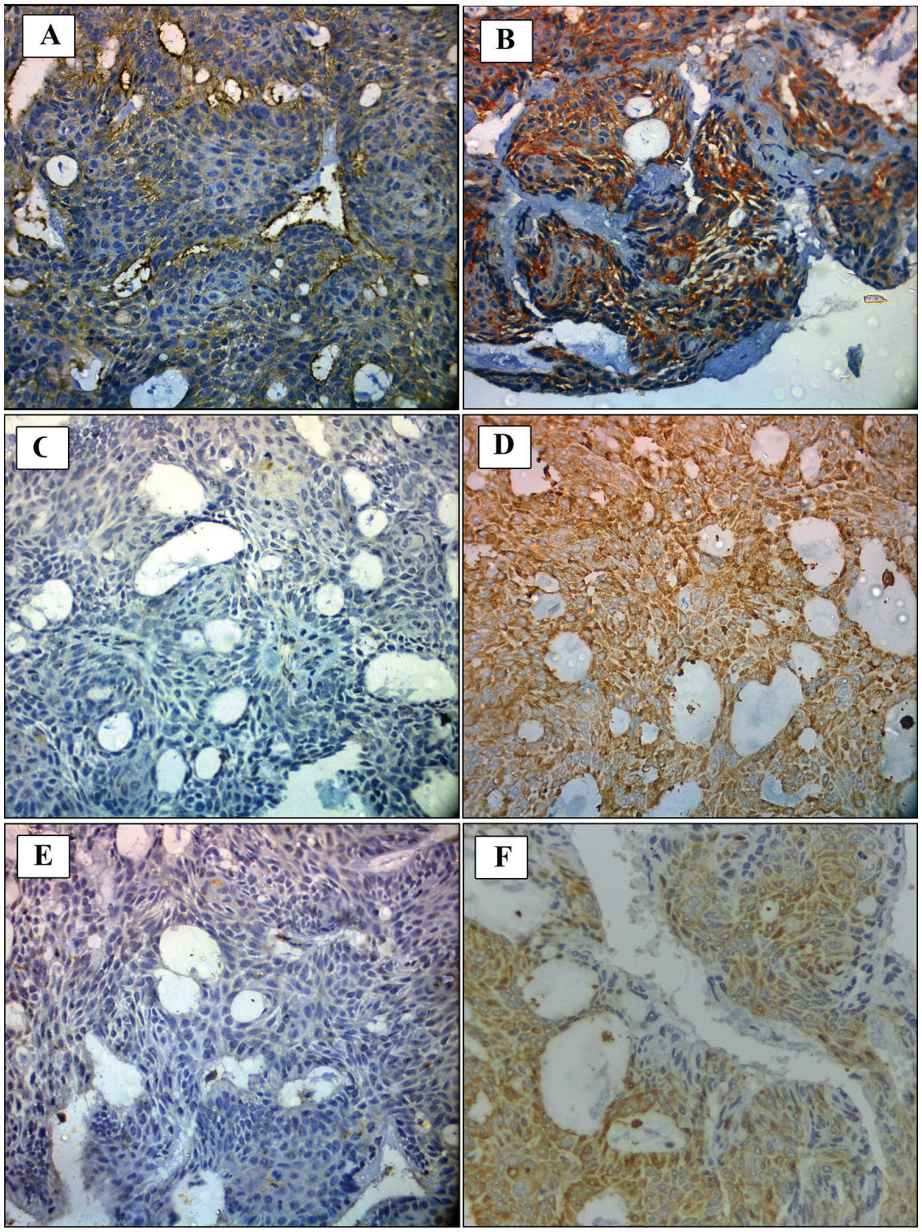

Figure 1: Immunohistochemistry of vascular endothelial growth factor (VEGF) shows (A) negative expression and (B) positive cytoplasmic expression in human papillomavirus-related multiphenotypic sinonasal carcinoma (HMSC) (×400). Immunohistochemistry of Bcl-2-associated X protein (BAX) shows (C) negative expression and (D) positive cytoplasmic expression in HMSC (×400). Immunohistochemistry of epidermal growth factor receptor (EGFR) shows (E) negative expression and (F) positive cytoplasmic expression in HMSC (×400).

was significantly associated with marked tumor size (P=0.010) and ≥45 age group (P<0.001). Relapse was significantly associated with high expression of BAX compared to patients with low expression (P=0.002). The five-year DFS was significantly higher in patients with low expression of BAX (87.5%) than those with high expression (32.9%) (P=0.035).

***EGFR*:** Of the 40 patients, 29 (72.5%) were EGFR-positive, and only 11 (27.5%) were EGFR-negative (figures 1E and 1F). The expression of EGFR-positive was significantly associated with advanced tumor stages III and IV (P=0.004), lymph node metastasis (P=0.004), tumor size (P=0.001), and ≥45 age group (P<0.001). Relapse was significantly associated with EGFR-positive compared to patients with EGFR-negative (P<0.001). The five-year DFS in patients with EGFR-negative was significantly higher than those with EGFR-positive (90.9% vs. 22.1%).

***ProEx™C*:** We observed positive staining for ProEx™C in the samples of 26 (65%) of the 40 patients, and 14 (35%) stained negative (figures 2A to 2D). The expression of ProEx™C was significantly associated with ≥45 age group (P<0.001), tumor size (P<0.001), advanced tumor stages III and IV (P<0.001), and lymph node metastasis (P<0.001). Relapse was

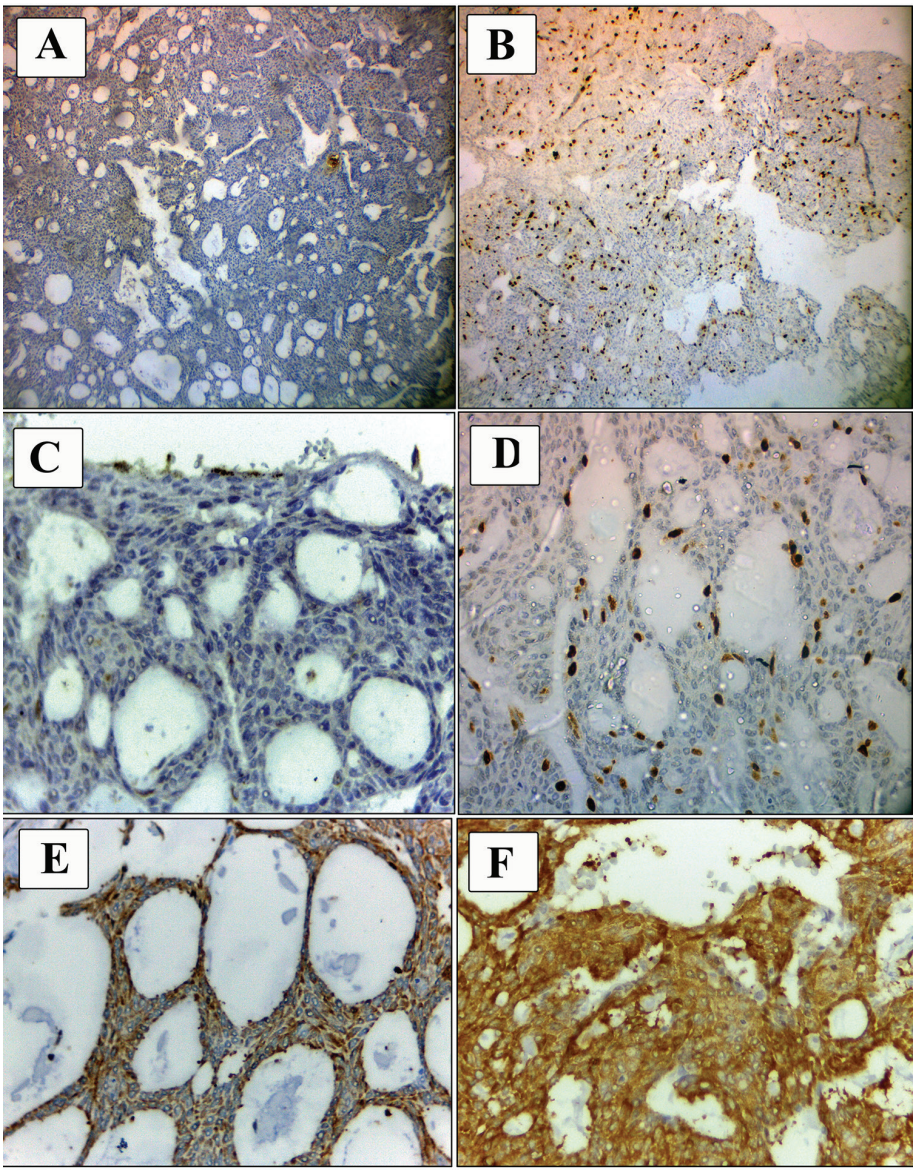

Figure 2: Immunohistochemistry of proEx™C shows (A) negative expression and (B) positive nuclear expression in HMSC (×100). High magnification (×400) images of (C) negative expression and (D) positive nuclear expression of proEx™C in HMSC. Immunohistochemistry of human telomerase reverse transcriptase (hTERT) shows (E) negative expression and (F) positive cytoplasmic expression in HMSC (×400).

significantly associated with positive expression of ProEx™C compared to patients with negative expression (P<0.001). The five-year DFS in patients with negative expression of ProEx™C (92%) was significantly higher than those with positive expression (13%) (P<0.001). The five-year OS was higher in patients with negative expression of ProEx™C (91.7%) than those with positive expression (56.1%) (P=0.02). Positive expression of ProEx™C was significantly associated with mortality (P=0.021).

hTERT: Of the 40 patients, 22 (55%) showed high expression, and 18 (45%) had low expression of hTERT (figures 2E and 2F). High expression of hTERT was significantly associated with lymph node metastasis (P<0.001), ≥45 age group (P<0.001), tumor size (P<0.001), and advanced tumor stages III and IV (P<0.001). Relapse was significantly associated with high hTERT expression compared to patients with low expression (P=0.001). The five -year DFS in patients with low expression of hTERT (75%) was significantly higher than those with high expression (15%) (P<0.001). Besides, the five -year OS was higher in patients with low expression of hTERT (94.1%) than those with high expression (47.7%). Furthermore, there was a significant association between high expression of hTERT and mortality (P=0.02).

Discussion

The results showed that the expression of VEGF, hTERT, and ProEx™C was significantly

associated with age, advanced tumor stages III and IV, lymph node metastasis, tumor size, relapse, poor DFS, poor OS, and mortality. Moreover, the expression of BAX was significantly associated with tumor size, age, poor DFS, and relapse (0.01, <0.001, 0.035, and 0.002, respectively).

HMSC is an HPV-related tumor with several histological features of ACC without MYB, MYBL1, or NIFIP translocation. While HMSC has squamous differentiation properties, it exhibits myoepithelial proliferation. Despite its aggressive behavior and high local recurrence rate, little is known about the clinical characteristics of HMSC. Therefore, an in-depth understanding of the biological processes involved in its development is needed to clarify its clinical characteristics and develop more effective therapeutic agents.[23]

In the present study, we diagnosed 40 patients with HMSC. Clinical presentation included polypoid tumors in the nasal cavity, nasal sinuses, and orbits that led to epistaxis, nasal obstruction, nasal discharge, pain, and visual symptoms.[10] Both sexes were affected, but in line with a previous study, women were in the majority. However, another study reported that only women were affected.[20] Of the 40 patients in our study, 20 (50%) had local recurrence and 2 (5%) had metastatic spread, which was consistent with a previous study reporting 36.4% and 4.5%, respectively.[23] Two other studies reported late recurrences with a follow-up of 50 months.[5, 24] However, Rupp and colleagues observed no recurrences or metastases in four patients with HMSC.[20]

One of the diagnostic criteria of HMSC is its association with HPV. We found that HPV-33 was present in 85%, HPV-35 in 10%, and HPV-16 in 5% of our patients. Other characteristics of HMSC are the lack of MYB/MYBL1 translocation, which distinguishes it from ACC, and the presence of myoepithelial differentiation, which distinguishes it from sinonasal SCC.[10] The morphology and HPV types (16, 33, and 35) in our patients were similar to those reported in previous studies.[10, 20, 21] However, Rupp and colleagues reported that one patient with HMSC had different morphology, i.e., glomerular patterns, more pleomorphic cells, and HPV-82.[20] Although the exact origin of cell in HMSC is unknown, the expression of pan-cytokeratin combined with biphasic staining of basal and myoepithelial proliferation, as well as their morphological characteristics, are indicative of the salivary gland origin.[10]

The majority of our patients (75%) showed high expression of VEGF, which was significantly associated with tumor size, age, advanced tumor stages, lymph node metastasis, recurrence, and mortality. Our results are in line with those of a previous study reporting that 71% of their patients had high expression of VEGF in ACC of the salivary gland.[22] Another study reported that the expression of VEGF was associated with poor prognosis of ACC, and its high expression was associated with advanced tumor stages, local recurrence, and poor OS.[25] However, Lee and colleagues observed that VEGF expression was not associated with survival rate or recurrence of ACC of salivary glands.[22]

In the present study, BAX was strongly expressed in 32 (80%) patients and was significantly associated with tumor size, age, relapse, and mortality. Our results were consistent with previous studies reporting high expression of BAX in 83% of their patients and its association with poor survival.[26, 27] Our results also showed that 29 (72%) patients were EGFR-positive, and its expression was significantly associated with tumor size, age, lymph node metastasis, and recurrence. These are in line with the results of a previous study reporting that 77% of their patients with ACC of salivary glands were EGFR-positive, and the expression was associated with poor prognosis.[24] In our patients, 65% tested positive for ProEx™C, which was significantly associated with tumor size, age, advanced tumor stages, lymph node metastasis, recurrence, and mortality. This is consistent with the conclusions of previous studies reporting that high expression of minichromosome maintenance protein 2 (MCM2) was associated with poor prognosis and advanced stages of salivary gland carcinomas.[28, 29] In contrast, another study reported that the downregulation of Ki-67 and MCM2 is associated with advanced tumor stages.[30] In line with the outcome of a study by Shigeishi and colleagues,[31] our results showed that 22 (55%) patients had high expression of hTERT, and it was strongly associated with tumor size, age, advanced stages, lymph node metastasis, recurrence, and mortality. Overall, our results indicated that the expression of VEGF, BAX, EGFR, hTERT, and ProEx™C was significantly associated with poor prognosis of HMSC. Large-scale prospective studies with more in-depth molecular assessments are recommended to substantiate our findings.

Conclusion

HMSC was strongly associated with HR-HPV. The expression of VEGF, BAX, EGFR, hTERT, and ProEx™C was shown to be associated with poor survival and aggressive malignant behavior,

making them novel prognostic biomarkers for targeted therapies in HMSC.

Authors' Contribution

All authors have equally contributed to the study conception and design; data acquisition, analysis, and interpretation; and drafting and revising the manuscript. All authors have read and approved the final manuscript and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Conflict of Interest: None declared.

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Original Article

Correlation of US-7 and US-9 Scores with Disease Activity Score using 28 Joint Counts (DAS28) in Patients with Rheumatoid Arthritis

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What's Known

- The German 7-joint Ultrasound, as a sonographic method, and Disease Activity Score Using 28 Joint Counts, as a clinical method, are the commonly used scoring systems in RA patients.

What's New

- Both the German 7-joint Ultrasound and the novel ultrasound score-based system, US-9 (joints assessed with the German 7-joint Ultrasound plus knees) scores are significantly correlated with the Disease Activity Score Using 28 Joint Counts score. However, there is no significant difference between them. Therefore, adding large joints such as knees into sonographic evaluation does not necessarily improve diagnostic value and clinical correlation.

Abstract

Background: The attentive management of rheumatoid arthritis (RA) has attracted particular attention. The German 7-joint Ultrasound (US-7) is the first scoring system that combines bone erosions and soft tissue lesions in a single composite scoring system. This study aimed to assess the correlation between US-7 and Disease Activity Score Using 28 Joint Counts (DAS28) in clinically active RA patients. The efficacy of a novel ultrasound score-based system, the US-9 score (joints assessed with US-7 plus knees), was also compared with the standard US-7 score.

Methods: All the RA patients referred to the outpatient rheumatology clinic of Ghaem Hospital, Mashhad, Iran, during 2019-2020 were included. 28 joints were clinically examined to calculate DAS28. Nine joints were assessed comprising the German US-7 plus knees using grayscale ultrasonography (GSUS) and power Doppler ultrasonography (PDUS). Retrieved data were analyzed by SPSS software, version 22. The Spearman Correlation test was used to find the correlation between DAS28 and ultrasonographic findings. The statistical significance level was set at $P<0.05$.

Results: This study was composed of thirty-five RA patients with a mean age of 49.1±12.0 years. US-7 synovitis scores in GSUS and PDUS were significantly correlated with DAS28 (P=0.02, r=0.38 and P=0.003, r=0.48, respectively). US-9 synovitis scores in GSUS and PDUS were also significantly correlated with DAS28 (P=0.003, r=0.49 and P=0.006, r=0.45, respectively). The synovitis score measured by GSUS was significantly correlated with the GSUS knee synovial score (P=0.01, r=0.42).

Conclusion: Ultrasound assessment of large joints such as knees can be an effective approach to determining RA severity. However, it can be proposed that adding more involved joints into the sonographic assessment does not necessarily provide a better clinical correlation.

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Keywords ● Arthritis, rheumatoid ● Ultrasonography ● Joints ● Ultrasonography, doppler

Introduction

Considerable progress has been made in our understanding of the underlying pathophysiology and therapeutic approaches of rheumatoid arthritis (RA). Therefore, significant concerns about

early diagnosis, attentive management, and treatment of RA have been raised.[1, 2] Clinical and imaging scoring systems are developed to monitor clinical findings, disease activity, and treatment response. Indeed, these modalities are used to achieve earlier diagnosis and adjust immunosuppressive therapy in RA patients.[3-5]

Disease activity score using 28 joint counts (DAS28) is a useful tool for measuring RA disease activity.[6] This scoring system was developed by Prevoo and others in 1994.[7] DAS28 evaluates 28 joints, including shoulders, elbows, wrists, metacarpophalangeal, proximal interphalangeal, and knee joints. Some recent studies have suggested limitations regarding the DAS28, such as identifying some patients as remitted cases despite the presence of subclinical disease activity, which causes erosive radiographic advancement and joint deformity.[8, 9]

Therefore, more accurate imaging modalities are needed to detect joint destruction at its initiation stage. Ultrasound (US) is one of the most sensitive imaging modalities for evaluating anatomical changes, such as synovitis, tenosynovitis, and bursitis, as well as bone lesions and treatment efficacy in patients with RA.[10, 11]

The German 7-joint Ultrasound (US-7) score, is a feasible joint scoring system, which is implemented for the assessment of disease severity in RA patients using ultrasound imaging.[12] The US-7 score evaluates joint lesions, including synovitis, tenosynovitis, and bone erosions based on seven joints of the clinically dominant hand and foot.[13] However, no US score systems such as US-7 have been established for large joint involvement in RA patients. The current study aimed to assess the value of US-7 as a marker of disease activity and evaluate its correlation with DAS28 in Iranian patients with RA. A new ultrasound score based on nine involved joints (joints assessed with US-7 plus the knees) (US-9) was also introduced and compared with the standard US-7 score.

Materials and Methods

Patients

In this cross-sectional study, after institutional review board approval (ethical code: IR.MUMS.fm.REC.1397.408), all RA patients who were referred to the outpatient rheumatology clinic of Ghaem Hospital, Mashhad, Iran, during 2019-2020, were assessed. All patients were diagnosed by an experienced rheumatologist according to the 2010 American College of Rheumatology/European League Against Rheumatism (ACR/EULAR) criteria.[14] After the confirmation of RA diagnosis (<7 days), the patient's demographic and socioeconomic data were recorded.

DAS28 was used to measure the disease activity. According to the DAS28, 28 joints, including proximal interphalangeal (PIP) and metacarpophalangeal (MCP), wrists, elbows, shoulders, and knees, were clinically evaluated by a rheumatologist for swelling or tenderness. DAS28 score was calculated by the formula on the DAS website (URL: https://www.das-score.nl/das28/DAScalculators/dasculators.html), with erythrocyte sedimentation rate (ESR), patient global health (0-100), and number of swollen and tender joints. Then, eligible patients who were not in the remission phase of RA (DAS28>2.5) signed the informed consent. Patients who had joint pain due to other etiologies, such as trauma, psychosomatic reasons, and degenerative diseases, were excluded from the study.

Ultrasonography

In the next step, patients were referred to the radiology inpatient service of Ghaem Hospital, Mashhad, Iran. They were examined with B-mode (grayscale) and power Doppler by a single radiologist with about seven years of experience who was blind to patients' DAS28 scores and disease severity. The selected joints of the wrist, hand, foot, and knees in all patients were evaluated by ultrasound according to the European League Against Rheumatism (EULAR).[15] Based on the German US-7, the MCP2, MCP3, PIP2, and PIP3 joints in the palmar and dorsal sides, the MTP2 and MTP5 joints in the dorsal and plantar sides, and the wrist joints in the palmar, dorsal, and ulnar sides were examined using grayscale ultrasonography (GSUS) and power Doppler ultrasonography (PDUS).

The method of German US-7 using GSUS and PDUS was previously described by Backhaus and colleagues.[13] In summary, GSUS was used to evaluate joint lesions, including bone erosions, tenosynovitis, and synovitis. Patients were scored based on the presence (1) or absence (0) of the tenosynovitis and erosions. Erosion was defined as the presence of interruption on the bone surface in two perpendicular planes. Synovitis was scored semi-quantitatively (grade 0: absence, grade 1: mild, grade 2: moderate, and grade 3: severe synovitis). PDUS was used to assess tenosynovitis and synovitis from the palmar and dorsal planes in each joint. Synovitis and tenosynovitis were scored semi-quantitatively (grade 0: no synovial color, grade 1: one or two small synovial color signals, grade

2: less than 50% of synovium filled with color signal, and grade 3: more than 50% of synovium filled with color signal).[16]

US-7 and US-9 Calculation

The sum of the GSUS and PDUS scores of each joint side was used to calculate US-7 score in five categories, including synovitis score by GSUS (score 0-27), tenosynovitis score by GSUS (score 0-7), erosion score by GSUS (score 0-14), synovitis score by PDUS (score 0-39), and tenosynovitis score by PDUS (score 0-21). To calculate the knee synovial score, PDUS and GSUS were performed and scored (0 to 3) in the medial, lateral, and superior compartments of both knees, separately. The semi-quantitative GSUS scoring of each knee compartment was as follows: grade 0=synovial thickness between 0- and 1.9-mm, grade 1=synovial thickness between 2- and 3.9-mm, grade 2=synovial thickness between 4 and 5.9 mm, and grade 3=synovial thickness about 6 mm and above. The sum of all three compartments' scores in both knees was assumed as GSUS knee synovial score (score 0-18).

The semi-quantitative PDUS scoring of each knee compartment was as follows: grade 0=no synovial color signal, grade 1=one or two small synovial color signals, grade 2=less than 50% of synovium filled with color signal, grade 3=more than 50% of synovium filled with color signal. The sum of all three compartments' scores in both knees was assumed as PDUS knee synovial score (score 0-18). The acquired scores from the knees were added to the measured GSUS and PDUS US-7 scores. The sum of the US-7 and knee synovial scores was assumed as the US-9 GSUS score (score 0-45) and the US-9 PDUS score (score 0-57).

Sample Size Calculation

As this study was the first that assessed the correlation between the newly introduced US-9 and DAS 28, and there was no previous article using US-9, the estimated sample size according to the possible correlation coefficient of 0.50 between US-9 and DAS 28 was calculated as 29. Since we also examined the correlation between US-7 and DAS 28, our sample size was estimated based on the data given in the article published by Leng and colleagues.[10] In the mentioned article, the correlation coefficient between DAS28 and US7 at the week sixth was reported as 0.45. Considering r=0.45, α=0.05, and β=0.2, the sample size was calculated as 36 individuals using the following formula:
$C=0.5*\ln[(1+r)/(1-r)]=0.4847$.
The standard normal deviate for $\alpha=Z\alpha=1.9600$
The standard normal deviate for $\beta=Z\beta=0.8416$
(Considering r=0.45, α=0.05, β=0.2)

The mentioned formula is accessible at the following address: http://sample-size.net/correlation-sample-size/

Statistical Analysis

All the statistical analyses were performed using the SPSS for Windows TM, version 20 software package (SPSS Inc., Chicago, IL, USA). Quantitative data were represented as mean±SD. For categorical variables, frequency and percentage were used. Bivariate correlations were performed using Spearman's Correlation test. A $P<0.05$ was considered as statistically significant.

Results

This study was composed of 35 RA patients with a mean age of 49.1±12.0 years (mean±SD). 33 patients were female and two were male. Patients' characteristics and demographic data are shown in table 1. The duration of the disease and the treatment ranged from 0 to 30 years and 0 to 20 years, respectively. The most common comorbidity was hypothyroidism (26.5%). Prednisolone, sulfasalazine, and methotrexate (MTX) were the most commonly used medications by patients. The DAS28 mean±SD was 6.1±1.2, and most patients had severe disease activity (85.7%).

Table 2 summarizes the PDUS and GSUS findings based on the US-7. GSUS and PDUS synovitis scores (US-7) were significantly correlated with DAS28 (P=0.02 and 0.003, respectively) (figures 1-4). Likewise, GSUS and PDUS knee synovial scores showed a statistically significant correlation with DAS28 (P=0.007 and 0.02, respectively). However, no significant correlation between DAS28 and tenosynovitis scores was found in the US-7 scoring system (P=0.16 for GSUS and P=0.19 for PDUS). There was also a significant correlation between synovitis score measured by GSUS (US-7) and GSUS knee synovial score (P=0.01, r=0.42). Both GSUS-9 (GSUS synovitis score [US-7] plus knee synovial GSUS score) and PDUS-9 (PDUS synovitis score [US-7] plus knee synovial PDUS score) scores were significantly correlated with DAS28 (P=0.003 and 0.006) (figure 5 and table 3).

Discussion

In this study, a significant correlation between both US-7 GSUS and PDUS and DAS28 was found. Likewise, there was a significant

Table 1: Patients' characteristics

Variable		Patients (N=35)
Age (year, mean±SD)		49.1±12
Sex N (%)	Male	2 (5.7%)
	Female	33 (94.3%)
Duration of disease (month, mean±SD)		86.17±81.94
Duration of treatment (month, mean±SD)		76.34±67.28
Comorbidities N (%)	Hypothyroidism	9 (26.5%)
	Hypertension	7 (20.6%)
	Hyperlipidemia	4 (11.8%)
	Diabetes mellitus	2 (5.9%)
ESR (mm/h, mean±SD)		33±21
DAS28 Score (mean±SD)		6.1±1.2
DAS28 grading N (%)	Moderate (3.2-5.1)	5 (14.3%)
	Severe (>5.2)	30 (85.7%)
Pharmacological treatment N (%)	Methotrexate	29 (89.2%)
	Prednisolone	29 (89.2%)
	Sulfasalazine	29 (89.2%)
	Calcium	21 (60%)
	Adalimumab	3 (8.6%)
	Hydroxychloroquine	4 (11.4%)

SD: Standard deviation; ESR: Erythrocyte sedimentation rate; DAS28: Disease activity score using 28 joint counts

Table 2: Ultrasonographic findings and scores in the study

	Variable	Score
GSUS	Tenosynovitis score (0-7)	1.3±0.7
	Synovitis score (0-27)	10.4±4.7
	Knee synovial score (0-18)	4.2±3.9
	US-9 score (0-45)	14.6±7.4
PDUS	Tenosynovitis score (0-21)	10±2.2
	Synovitis score (0-39)	7.5±4.9
	Knee synovial score (0-18)	2.2±1.5
	US-9 score (0-57)	9±5.9

GSUS: Grayscale ultrasonography; US-9: 9-joint ultrasonography; PDUS: Power Doppler ultrasonography

Table 3: Correlation between DAS28 and ultrasonographic parameters

	Variable	P value*	Correlation Coefficients (r)
GSUS (US-7 score)	Synovitis score (US-7 score)	0.02	0.38
	Tenosynovitis score (US-7 score)	0.16	0.24
PDUS (US-7 score)	Synovitis score (US-7 score)	0.003	0.48
	Tenosynovitis score (US-7 score)	0.19	0.23
Ultrasonographic assessments of knees	GSUS knee synovial score	0.007	0.45
	PDUS knee synovial score	0.02	0.39
	GSUS synovitis score plus knee synovial GSUS score (US-9)	0.003	0.49
	PDUS synovitis score plus knee synovial PDUS score (US-9)	0.006	0.45

GSUS: Grayscale ultrasonography; US-7: 7-joint ultrasonography; PDUS: Power Doppler ultrasonography; US-9: 9-joint ultrasonography; *Spearman correlation test was used.

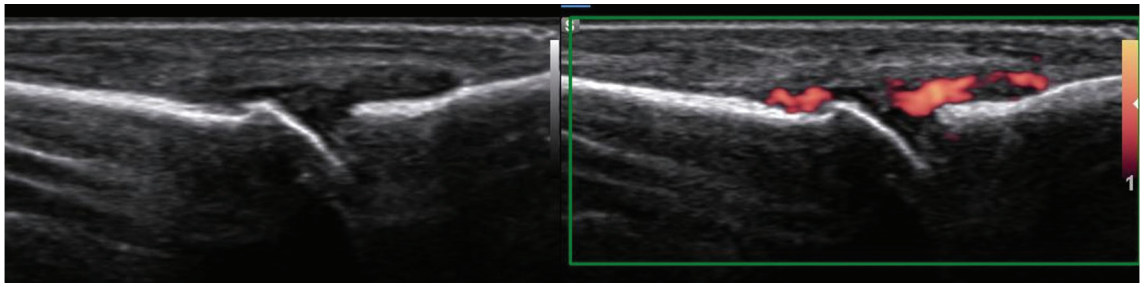

Figure 1: Grayscale ultrasonography (GSUS) and power Doppler ultrasonography (PDUS) of 3rd MCP joint dorsal side show grade 2 synovitis by GSUS and grade 3 synovitis by PDUS.

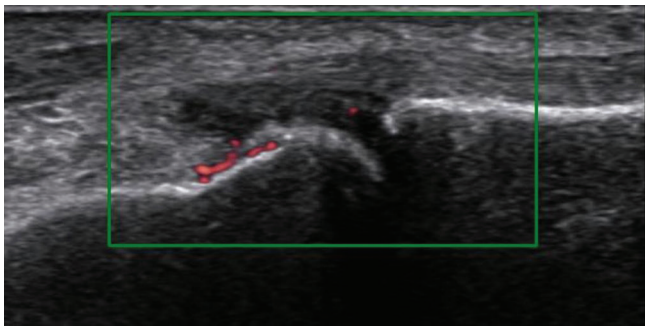

Figure 2: Grayscale ultrasonography (GSUS) and power Doppler ultrasonography (PDUS) of 2nd MCP joint palmar side show grade 3 synovitis by GSUS and grade 1 synovitis by PDUS.

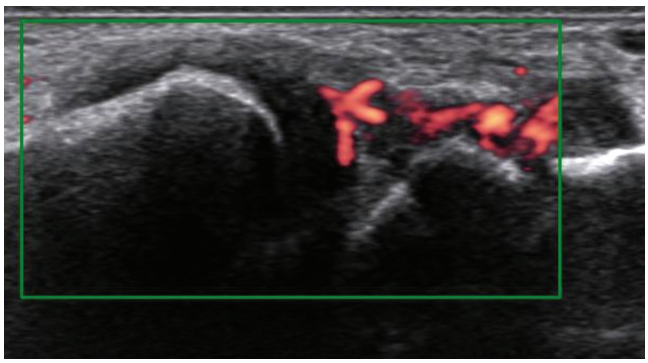

Figure 3: Grayscale ultrasonography (GSUS) and power Doppler ultrasonography (PDUS) of wrist ulnar side show grade 3 synovitis by GSUS and grade 2 synovitis by PDUS.

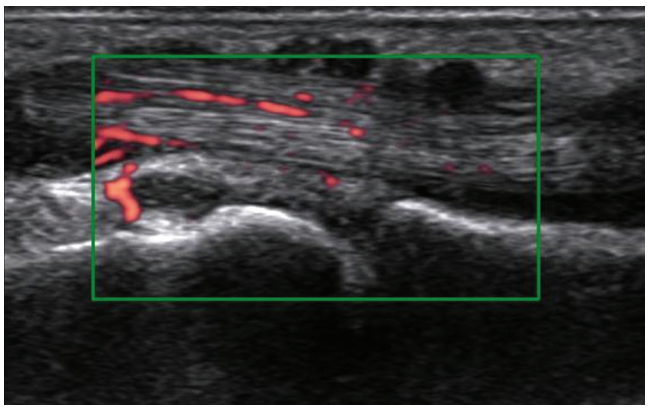

Figure 4: Grayscale ultrasonography (GSUS) and power Doppler ultrasonography (PDUS) of the 3rd MCP joint palmar side show tenosynovitis in the flexor tendon.

correlation between both US-9 GSUS and PDUS and DAS28. However, there was not a remarkable difference regarding their correlation with DAS28.

This study has been conducted to assess the utility of ultrasonography of large joints such as the knee joint in addition to the US-7 ultrasound joint scoring system in RA. To our knowledge, this study was performed for the first time on Iranian patients, as well as the Middle-Eastern population, assessing the correlation of the US-7 score with DAS28 in patients with active RA.

The US is one of the most sensitive and reliable approaches to manage and monitor disease activity. In addition, it helps to detect patients with low disease activity, as well as those who are in the remission phase based on clinical scoring systems such as DAS28. However, the role of the US in the management and treatment of RA is not clear yet.[8, 9]

A large body of evidence investigated (qualitatively and quantitatively) synovitis by Doppler in patients with RA.[17-20] Among proposed US-based approaches, US-7 is one of the most feasible and accurate methods.[11] In the current study, we used the German US-7 out of several available US-based scoring systems due to its accuracy and feasibility.[21, 22] US-7 was the first scoring system that combined bone erosions as well as soft tissue lesions, such as synovitis and tenosynovitis, in a single composite scoring system. Besides, since US-7 can be performed

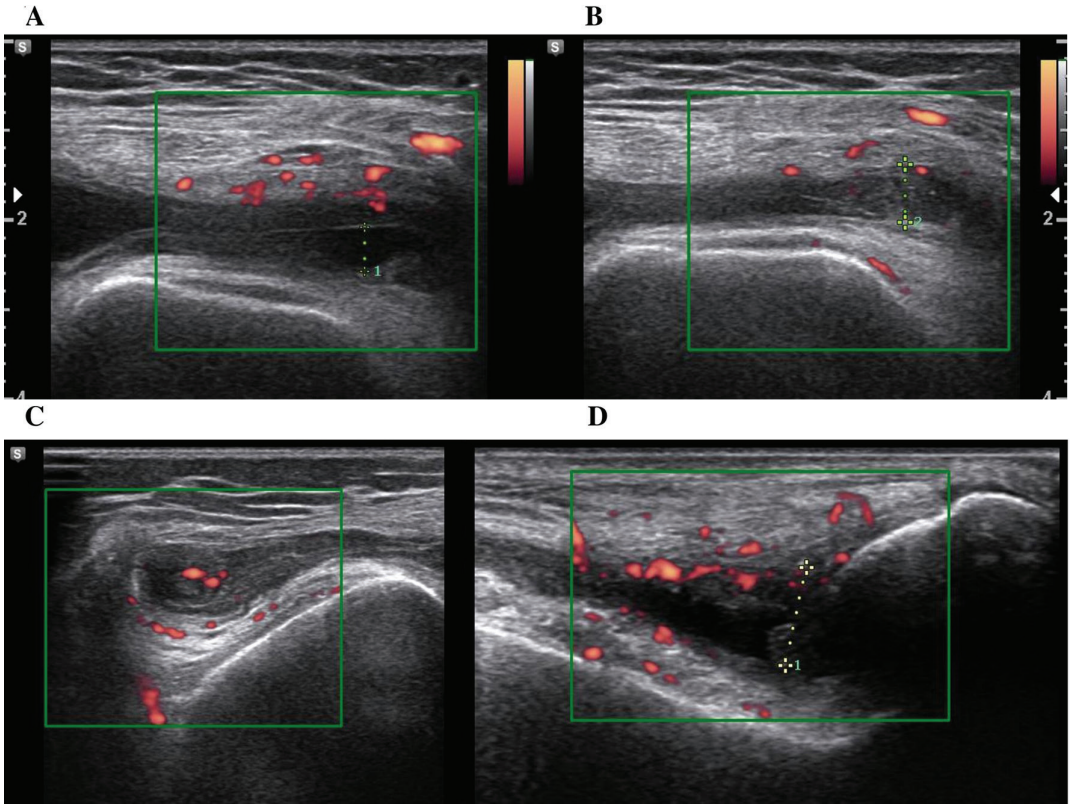

Figure 5: Grayscale ultrasonography (GSUS) and power Doppler ultrasonography (PDUS) measurements of the knee. (A) Shows effusion measurement with GSUS in the medial compartment. (B) shows the same region after compression with grade 1 synovitis by PDUS and GSUS, (C) grade 2 synovitis by GSUS and PDUS, and (D) Grade 3 synovitis by PDUS.

with a limited number of joints (seven joints), assessment with US-7 took approximately 10–20 min for each patient, which is very critical in daily radiologic practice.[22] The possible correlation between DAS28, as a clinical scoring system, and US-7 was investigated. A significant positive correlation was found between DAS28 and synovitis scores in the US-7 scoring system measured by GSUS and PDUS. No significant correlation was found between DAS28 and tenosynovitis and erosion scores in the US-7 scoring system.

The PDUS and GSUS of both knees were also assessed and the correlation of our new approach, (US-9), with the clinical assessment using DAS28 was evaluated. A significant correlation was found between knee compartments' synovial thickness and DAS28 score and US-7 synovitis score. This indicates that ultrasound assessment of large joints such as knees can be an effective approach to determine RA severity. According to our findings, US-7 and US-9 revealed similar results. Therefore, including large joints such as the knees in the sonographic assessment does not necessarily provide better diagnostic performance in the assessment of disease activity.

Our results are in agreement with previous reports that indicated the significant positive correlation between DAS28 and synovitis score in US-7 using GSUS and PDUS.[11, 13, 23, 24] US is a more sensitive modality than clinical assessments in the detection of synovitis.[22] PDUS can assist in distinguishing actively inflamed joints from inactive joints, which is an important issue in RA management.[16] PDUS can also identify patients with well-controlled disease[25] and predict relapse within one year in RA patients classified as clinical remission based on DAS28.[26] In another study, US-7 is shown to be sensitive to change over a treatment period.[10] The DAS28 scoring system requires some experience by the rheumatologist and is time-consuming in some cases. Thus, the US would be utilized in addition to DAS28 to achieve better differentiation between active and remitted phases of RA disease.

The association between clinical disease activity indices and US-7 was evaluated in previous studies.[27, 28] Mahesh Wari and his colleagues studied sixty-two RA patients with a mean age of 44 years and a median treatment duration of 7 years. In line with our results, they found a significant correlation between DAS28 and synovitis by GSUS and PDUS scores. Similar to our results, they did not find any correlation between DAS28 and erosion score and tenosynovitis on GSUS and PDUS.[11]

Kamel and his colleagues studied fifty RA patients in a cross-sectional study with a mean disease duration of 8.7 years. They reported that DAS28 scores were positively correlated with synovitis scores on GSUS and PDUS assessed by US-7.[29]

Although DAS28 is still a common method for measuring RA activity, its application has been argued recently due to some drawbacks.[8] For instance, Terslev and coworkers detected subclinical synovitis using US in the majority of RA patients in longstanding DAS28-remission.[9] There are some other scoring systems for measuring RA activity. Kamel and others evaluated the association between the US-7 and disease activity, based on the Clinical Disease Activity Index (CDAI), global arthritis score (GAS), and the Routine Assessment of Patient Index Data 3 (RAPID 3). All three disease activity indices were found to be significantly correlated with GSUS and PDUS synovitis.[29] Future studies can evaluate the correlation between the US-7 scoring system and other clinical disease activity indices.

In the present study, both US-7 and US-9 were significantly correlated with DAS28, but there was no significant difference between these two US methods. Leng and colleagues evaluated US-7 and US-12 in RA patients and reported no significant difference regarding response to therapy.[10] However, new scoring methods may be discovered by further exploration that differ significantly from the previously established systems, regarding disease activity and treatment response.

Our study had some limitations, including the lack of follow-up examinations for the patients to assess the possible alterations in our study variables. We could also use other clinical scoring systems in addition to DAS28. However, based on the literature, DAS28 is the most commonly used method for assessing RA patients in clinical studies and daily practice.[3, 30] RA is more prevalent in women, and they usually experience a more severe form of RA.[31] Consistently, most of the patients were female (94.3%) in the current study. It is recommended to compare the utilization of US-7 in both men and women in future studies. We hope that a new study with more patients may lead to more accurate results.

Conclusion

The US-7 is a simple, accurate, and practical scoring system to predict disease activity in RA patients. Knee ultrasound alone is also effective in determining the severity of the disease. However, the combination of them and adding large joints' scores such as knees, do not improve its diagnostic value necessarily.

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Authors' Contribution

All authors contributed to the conception and design. M.E contributed to the analysis and interpretation of the data. S.E, A.M.A, H.R.H, and R.G contributed to drafting the article. M.S, M.E, F.K and B.A contributed to the critical revision of the article for important intellectual content. All authors have read and approved the final manuscript and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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Original Article

Disappearance of Imported Cases of Omicron Lineage BA.2.40 in West Kalimantan, Indonesia

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Abstract

Background: The World Health Organization has declared Omicron as the fifth variant of concern with more than 50 mutations, particularly in the spike protein. Given increased viral infectivity due to mutations, worldwide genomic surveillance and detection of severe acute respiratory syndrome 2 (SARS-CoV-2) is essential. The present study aimed to track Omicron lineage BA.2.40 in West Kalimantan, Indonesia.

Methods: In May-August 2022, nasopharyngeal swab samples (n=3,642) were collected from international travelers to West Kalimantan (active surveillance), and patients hospitalized due to SARS-CoV-2 infection (baseline surveillance). The samples were tested for Omicron lineages based on ORF1ab, N, and HV69-70del genes, followed by whole-genome sequencing. The sequences were then identified using two genomic databases, aligned against the reference genome (Wuhan/Hu-1/2019), and then compared with BA.2.40 lineage detected across the world. Phylogenetic analysis between the samples and other SARS-CoV-2 isolates was performed using molecular evolutionary genetics analysis software.

Results: Based on the genomic databases, 10 isolates were identified as BA.2.40. All samples tested positive for the ORF1ab and N genes, but negative for the HV69-70del gene, which is a marker to detect the Omicron variant. Phylogenetic analysis showed the isolates were closely related to an isolate from Malaysia, an area dominated by BA.2.40.

Conclusion: Omicron lineage BA.2.40 has no HV69-70 deletion in the spike protein, a marker used to screen for the Omicron variant. BA.2.40 showed a high similarity to an isolate from Malaysia and was detected only during certain periods, indicating the effect of internationally imported cases.

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Keywords ● SARS-CoV-2 ● Indonesia ● Mutation ● COVID-19 ● Spike glycoprotein, coronavirus

What's Known

- Omicron is declared a variant of concern due to its increased viral infectivity, higher transmission rate, and worldwide spread.
- Genomic surveillance of coronavirus disease-19 as a prevention and control strategy is essential, since genetic mutations are associated with disease severity and the spread of viral infections.

What's New

- BA.2.40 has no HV69-70 deletion in the spike protein, a marker used to screen for the Omicron variant. Therefore, the use of this marker as a screening tool should be re-evaluated.
- BA.2.40 in West Kalimantan (Indonesia) had the same origin as the variant in Malaysia, indicating the effect of internationally imported cases.

Introduction

Among all variants of severe acute respiratory syndrome 2 (SARS-CoV-2), on 24 November 2021, the World Health Organization (WHO) declared Omicron as the fifth variant of concern (VOC). Previously declared variants were Alfa, Beta, Delta, and Gamma. VOC is defined when a virus (e.g., SARS-CoV-2) mutates into variants that spread more easily, become more virulent, change clinical presentations, or reduce the effectiveness of available diagnostics, vaccines, and treatments.[1] So far, Omicron has 50

genetic mutations, of which 30 are spike protein mutations; making it more infectious but less severe than previous variants.[2]

Omicron is currently the dominant variant worldwide. It poses a global threat due to its high contagiousness compared to other variants, with 7.9 million genomic sequences recorded in the Global Initiative on Sharing All Influenza Data (GISAID) EpiCoV™ database.[3] Omicron variant (B.1.1.529) was first identified in South Africa, followed by lineages BA.1, BA.2, BA.3, BA.4, BA.5, and descendent sublineages.[1] BA.1, BA.1.1, BA.2, and their sublineages are considered the most important variants and are closely monitored. BA.2 has shown a higher transmission rate than BA.1, but with similar disease severity.[4]

Rapid mutation of SARS-CoV-2 variants has necessitated the use of genomic surveillance as the main approach to closely monitor the growth of circulating lineages. A comprehensive understanding of the SARS-CoV-2 genomic evolution serves as an early sign for epidemiological monitoring and provides information to policymakers who can then adjust their strategies to manage the pandemic. The socio-economic implications of the disease have been grave. Social distancing, travel restrictions, and lockdowns have severely affected public and private businesses, the transportation industry, and the employment rate. In Indonesia, thousands of people have lost their jobs due to the pandemic. Consequently, extensive travel back and forth by Indonesian workers between West Kalimantan and Malaysia. This in turn has affected the epidemiological pattern of the disease in Indonesia, especially in West Kalimantan, enforcing targeted genomic surveillance of international travelers. The present study aimed to track Omicron variant BA.2.40 in West Kalimantan from May to August 2022. The target population was suspected cross-border workers with symptoms of coronavirus disease-19 (COVID-19), and patients hospitalized due to the disease.

Materials and Methods

Both baseline and active surveillance of the disease were carried out. Active surveillance was conducted of travelers arriving at the international entry point of West Kalimantan with COVID-19 symptoms. Baseline surveillance was carried out on all patients hospitalized for SARS-CoV-2 infection. From May to August 2022, a total of 3,642 nasopharyngeal swab samples were collected and tested for SARS-CoV-2. Of these, 159 samples were screened for Omicron variants in the Laboratory of Microbiology, Tanjungpura University Hospital (Pontianak, Indonesia). All samples with cycle threshold (Ct) values below 33 were included in the study. Whole-genome sequencing was performed in the Department of Biochemistry and Molecular Biology, School of Medicine, Tanjungpura University Hospital. The study design and sample collection were approved by the Ethics Committee of Tanjungpura University Hospital (number: 8179/UN22.9/PG/2022).

Detection of Omicron Variant

DNA/RNA extraction kit was used to extract viral DNA/RNA from nasopharyngeal swab samples using GeneRotex 96 rotary nucleic acid extractor (Tianlong Science and Technology, Xi'an, China). RNA extraction was performed using 200 uL of samples to extract 60 uL of total RNA. RNA amplification was performed using QuantStudio™ 5 real-time polymerase chain reaction (RT-PCR) system (Applied Biosystems, Thermo Fisher Scientific, Massachusetts, US). The PCR test was performed using a total volume of 25 uL to detect three target genes, namely ORF1ab, N, and HV69-70del. The P732H multiplex detection kit (Tianlong Science and Technology, Xi'an, China) contained 12.5 uL reaction solution, 1 uL enzyme mix, 6.5 uL primer and probe mix, and 5 uL RNA negative and positive controls. ORF1ab is the common gene in the Beta variant and is widely used in SARS-CoV-2 detection due to its high sensitivity, specificity, and positive predictive value.[5] The nucleocapsid (N) protein protects the viral genome and is commonly used in COVID-19 detection.[6] HV69-70del gene is one of the key features of Omicron lineage B.1.1.7, and is widely used to differentiate B.1.1.7 from other lineages.[7, 8]

The results of Omicron screening were categorized into two groups, namely probable samples (positive result on ORF1ab, N, and HV69-70del genes) and nonprobable samples (negative result on HV69-70del gene). Both probable and nonprobable samples were further analyzed using whole-genome sequencing.

Library Preparation and Sequencing

RNA library preparation for sequencing was performed using Rapid Barcoding kit SQK-RBK110.96 and Midnight RT-PCR expansion kit EXP-MRT001 (Oxford Nanopore Technologies; Oxford, UK). The input volume for each sample was 8 µL. After barcoding the reaction in each well plate, all samples were pooled and purified with solid-phase reversible immobilization (SPRI) beads using a microcentrifuge tube

magnetic separation rack. One microliter of rapid adapter (RAP) was added to 10 µL of the pooled eluate (800 ng), and the library was kept on ice until loaded onto the flow cell. A total of 75 µL solution contained 12 µL DNA library, 37.5 µL sequencing buffer, and 25.5 µL loading solution.

The sequencing was performed using a MinION sequencer (Oxford Nanopore Technologies, Oxford, UK), and local base-calling was performed using the MinKNOW software. Demultiplexing and identification of SARS-CoV-2 were performed using the EPI2ME cloud-based bioinformatics platform (https://labs.epi2me.io/; Oxford Nanopore Technologies, Oxford, UK). The analysis was performed using Fastq QC+ARTIC+NextClade 2022.07.19-15399 with a default minimum Q-score threshold of 8. The MinION ran for up to six hours (28,000 sequence reads). The sequences were identified using Pangolin (https://pangolin.cog-uk.io/) and GISAID (https://gisaid.org/) databases.

Phylogenetic Sequence Analysis

The sequences were aligned against the reference genome (Wuhan/Hu-1/2019) and six other genomes (Beta B.1.351, Delta B.1, Gamma P.1, Alpha B.1.1.7, Lambda C.37, and Omicron B.1.1.529) using the MAFFT method.[9] The phylogenetic tree was constructed using the neighbor-joining (NJ) method with Molecular Evolutionary Genetics Analysis (MEGA X) software (Philadelphia, US) to visualize the evolutionary relationship between our original samples and other SARS-CoV-2 isolates. The distribution of the data from the GISAID database and isolates from the swabs was visualized on a world map. In addition, the mutation point of BA.2 was compared with Omicron, Omicron BA.5, Omicron BQ.1, Omicron BQ.1, Omicron XBB.1, Omicron BA.2.75, Alpha, Beta, Delta, and Gamma variants using a web-based application (https://outbreak.info/compare-lineages).

Results

Sample Characteristic and Surveillance Strategy

Of the 3,642 nasopharyngeal swab samples, 159 isolates were screened for Omicron followed by whole-genome sequencing analysis. Of these, based on pangolin and GISAID databases, 10 isolates were identified as Omicron lineage BA.2.40 (table 1). Except for isolate 01, all other isolates belonged to Indonesian workers traveling from Tebedu (Sarawak, Malaysia) to Entikong (West Kalimantan, Indonesia) between June and July 2022 (active surveillance). Isolate 01 was also Omicron lineage BA.2.40, but from a child hospitalized following RT-PCR testing. This patient was included in the baseline surveillance category due to age and low Ct value (table 2).

Table 1: Characteristics of isolates with Omicron lineage BA.2.40 in West Kalimantan

Isolates	Sex	Age (years)	Sampling date	Origin	Surveillance
01	Female	4	09-05-2022	Pontianak	Baseline
02	Male	21	23-06-2022	Sanggau	Active
03	Male	56	23-06-2022	Sanggau	Active
04	Female	51	23-06-2022	Sanggau	Active
05	Male	27	23-06-2022	Sanggau	Active
06	Male	25	29-06-2022	Sanggau	Active
07	Male	43	01-07-2022	Sanggau	Active
08	Male	39	01-07-2022	Sanggau	Active
09	Male	18	01-07-2022	Sanggau	Active
10	Male	24	01-07-2022	Sanggau	Active

Table 2: The results of real-time polymerase chain reaction and whole-genome sequencing for detecting targeted genes

Isolates	Ct values of targeted genes			Sample status	WGS
	ORF1ab	N	HV69-70del		
01	22.66	21.88	0.00	Nonprobable	BA.2.40
02	17.63	19.28	0.00	Nonprobable	BA.2.40
03	17.64	19.64	0.00	Nonprobable	BA.2.40
04	22.36	23.34	0.00	Nonprobable	BA.2.40
05	28.44	30.29	0.00	Nonprobable	BA.2.40
06	29.65	31.10	0.00	Nonprobable	BA.2.40
07	29.65	31.10	0.00	Nonprobable	BA.2.40
08	25.98	26.88	0.00	Nonprobable	BA.2.40
09	29.74	31.46	0.00	Nonprobable	BA.2.40
10	26.78	28.64	0.00	Nonprobable	BA.2.40

Ct: Cycle threshold; ORF: Open reading frame; N: Nucleocapsid; HV69-70del: Deletion at sites 69 (histidine) and 70 (valine); WGS: Whole-genome sequencing

Detection of Omicron Variant using RT-PCR and Whole-genome Sequencing

All samples tested positive for ORF1ab and N genes with Ct values ranging from 17.63 to 29.74 and 19.28 to 31.46, respectively. However, in contrast with Omicron lineage B.1.1.7, none of the samples showed HV69-70del mutation (Ct=0). Therefore, isolates without HV69-70del mutation were classified as nonprobable samples (table 2).

The search in the GISAID database did not reveal the emergence of BA.2.40 in other Indonesian provinces. Therefore, the search was extended to other countries. In line with a previous study, the results showed that of the variants deposited in GISAID, 124 isolates were from Malaysia, 11 from Australia, three each from Germany and USA, and one each from Austria, Nigeria, Colombia, Israel, Denmark, and Thailand.[10] Seven reference genomes were used, namely Wuhan-Hu-1, Beta (B.1.351), Delta (B.1), Gamma (P.1), Alpha (B.1.1.7), Lambda (C.37), and Omicron (B.1.1.529) variants. The final dataset included 30,326 positions. As shown in figure 1, all samples with BA.2.40 had originated from Omicron B.1.1.529. We also found that the origin of the identified isolate was very similar to the isolate from Malaysia. Based on the GISAID database, the first case of BA.2.40 was identified in Sarawak (Malaysia) in February 2022. Although BA.2.40 is widespread across the world, 81% of the cases were detected in Malaysia (figure 2). Whole-genome sequencing is still performed in Indonesia, however, the last detection of BA.2.40 dates back to July 1, 2022. The corresponding samples (n=4) belonged to those traveling from Malaysia (table 1).

Spike Protein Mutation in BA.2.40 from West Kalimantan

Genomic sequencing analysis of all isolates from West Kalimantan was performed and submitted to GISAID for data sharing and epidemiological data analysis. The sequencing coverage of all samples was in the range of 3.81-6.13%, with the exception of isolate 06 with 17% coverage. The results confirmed that the 10 isolates from West Kalimantan had 97.4% similarity with the lineage BA.2.40 compared to the reference strain hCoV-19/Wuhan/WIV04/2019, the first SARS-CoV-2 genome strain detected in Wuhan, China.

The results showed 31 mutations in the spike protein, of which 21 mutations in non-structural protein, 7 in N protein, 2 in membrane (M) protein, and a single mutation in NS and E proteins.[11] However, in this study, we only focused on mutation in the spike protein. Among the 31 mutations, three deletions occurred at sites 24, 25, and 26 for leucine and proline, respectively. As shown in table 3, other mutations caused a change in the amino acid of the spike protein. Most mutations were found in subunit 1 (26 site mutations), 16 of which were specifically found in the receptor-binding domain (RBD) of the spike protein, and

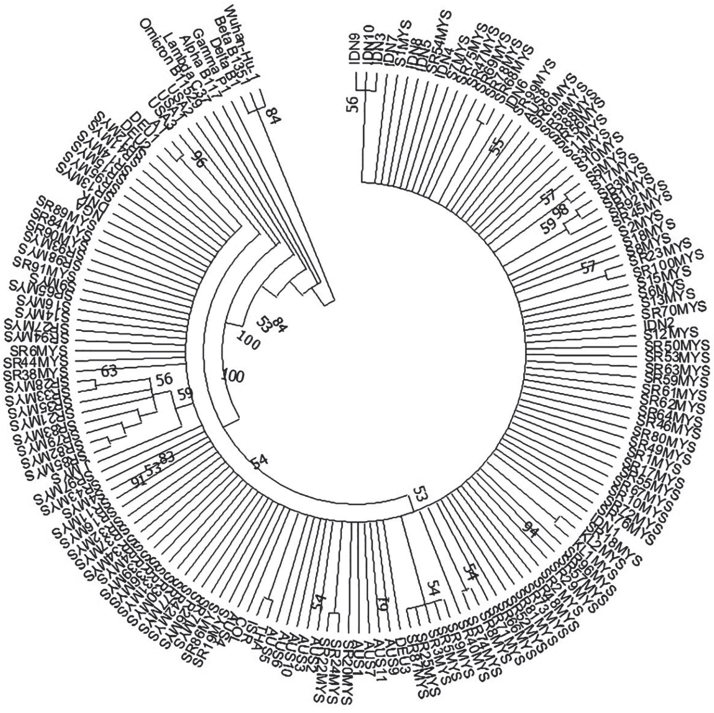

Figure 1: The Phylogenetic Tree of Indonesian BA.2.40 variants compared with all BA.24.40 variants from Malaysia (MYS), Australia (AUS), Germany (DEU), United States of America (USA), Austria (AUT), Nigeria (NGA), Colombia (COL), Israel (ISR), Denmark (DNK), and Thailand (THA)

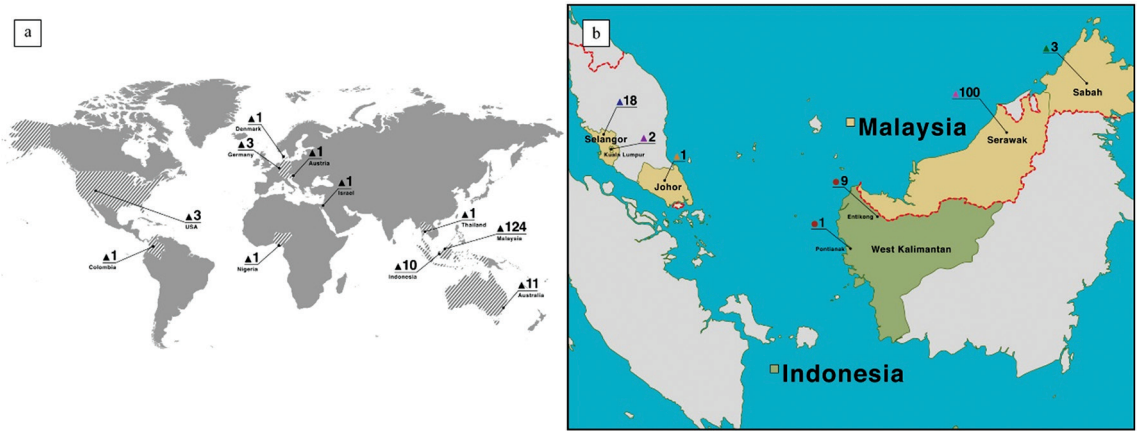

Figure 2: a) The worldwide distribution of BA.2.40 variants (data was retrieved from GISAID database) **b)** most of BA.2.40 varaints was found in Malaysia (as 6 December 2022)

Table 3: Spike protein mutations of Omicron lineage BA.2.40 in isolates from West Kalimantan

Number	Mutation	Mutation type	AA replacement		Occurrence based on GISAID*	
			Original AA	Mutated AA	Rate (%)	Number of affected countries
1	T19I	AA replacement	Threonine	Isoleucine	28.27	178
2	L24del	Deletion	-	-	27.93	176
3	P25del	Deletion	-	-	27.94	176
4	P26del	Deletion	-	-	27.95	176
5	A27S	AA replacement	Alanine	Serine	28.15	186
6	G142D	AA replacement	Glycine	Aspartic acid	67.12	211
7	V213G	AA replacement	Valine	Glycine	28.99	177
8	G339D	AA replacement	Glycine	Aspartic acid	45.04	207
9	S371F	AA replacement	Serine	Phenylalanine	28.14	179
10	S373P	AA replacement	Serine	Proline	44.25	205
11	S375F	AA replacement	Serine	Phenylalanine	44.13	205
12	T376A	AA replacement	Threonine	Alanine	28.18	178
13	D405N	AA replacement	Aspartic acid	Asparagine	28.56	179
14	R408S	AA replacement	Arginine	Serine	27.22	178
15	K417N	AA replacement	Lysine	Asparagine	39.88	207
16	N440K	AA replacement	Asparagine	Lysine	38.70	203
17	S477N	AA replacement	Serine	Asparagine	44.63	207
18	T478K	AA replacement	Threonine	Lysine	75.97	213
19	E484A	AA replacement	Glutamic acid	Alanine	44.12	206
20	Q493R	AA replacement	Glutamine	Arginine	31.16	200
21	Q498R	AA replacement	Glutamine	Arginine	43.12	204
22	N501Y	AA replacement	Asparagine	Tyrosine	53.23	213
23	Y505H	AA replacement	Tyrosine	Histidine	43.25	204
24	D614G	AA replacement	Aspartic acid	Glycine	99.23	216
25	H655Y	AA replacement	Histidine	Tyrosine	48.54	210
26	N679K(674)	AA replacement	Asparagine	Lysine	47.46	208
27	P681H(674)	AA replacement	Proline	Histidine	56.64	214
28	N764K	AA replacement	Asparagine	Lysine	44.64	207
29	D796Y	AA replacement	Aspartic acid	Tyrosine	46.80	206
30	Q954H	AA replacement	Glutamine	Histidine	45.53	205
31	N969K	AA replacement	Asparagine	Lysine	46.62	205

GISAID: Global initiative on sharing all influenza data; AA: Amino acid; *GISAID database per 6 December 2022.

5 mutations in subunit 2 (S2). Based on records retrieved from GISAID (as of December 6, 2022), the mutation rate in our samples ranged from 27.22% to 99.23%. Five mutations with a rate >50% were D614G (99.23%), T478K (75.97%), G142D (67.12%), P681H (56.64%), and N501Y (53.23%). The classification of SARS-CoV-2 variants was made based on the occurrence of a mutation. Omicron lineage BA.2 has similar mutations to other Omicron lineages, with the exception of the Q493R mutation and no HV69-70 deletion (figure 3).

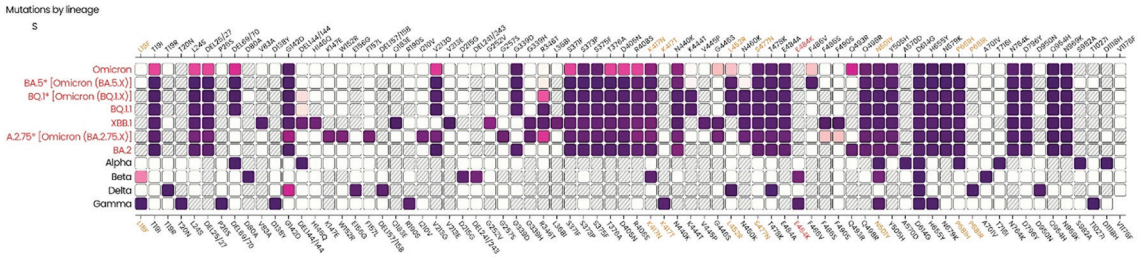

Figure 3: Comparison of Mutation Point of BA.2 with Omicron, Omicron BA.5, Omicron BQ.1, Omicron BQ.1, Omicron XBB.1, Omicron BA.2.75, Alpha, Beta, Delta, and Gamma Variants

Discussion

For the first time, genomic surveillance of SARS-CoV-2 was conducted in mid-2022 in West Kalimantan. By August 2022, we sequenced 159 isolates, of which 10 isolates were classified as Omicron lineage BA.2.40. This is the first time that cases of BA.2.40, a descendent of BA.2 lineage, have been reported from West Kalimantan. Although BA.2.40 is widespread in the world, only a total of 157 isolates across 11 countries were reported in the GISAID database.[11] Our genomic analysis showed that all samples with BA.2.40 were of the same parental lineage B.1.1.529, a VOC with 62 site mutations. Currently, Omicron is the dominant variant in the world, posing a serious threat due to its high rate of contagiousness compared to other VOC variants.[12] BA.1 and BA.2 lineages have similar disease severity. However, BA.2 is closely monitored by the WHO because of its high transmission rate.[4]

In 2022, we performed genomic surveillance of SARS-CoV-2, and 10 isolates were identified as BA.2.40, of which nine isolates were from Indonesian workers traveling from Sarawak (Malaysia) to West Kalimantan (Indonesia). In Malaysia, 124 isolates with BA.2.40 were detected from February to March 2022. The samples were collected mainly in Sarawak province, followed by Selangor, Sabah, and Johor provinces. The first cases of BA.2.40 in Indonesia were detected in West Kalimantan, but it had almost disappeared from the region by May 2022, with the last case reported on August 31, 2022. During the same period, based on our laboratory data deposited in GISAID, variant BA.5 followed by XBB became the dominant strain in the region.[10] These variants are more transmissible than the previous variants[13, 14] and also designated as VOC. This may explain why these two variants became dominant over other variants, including BA.2.40. A similar trend was also observed in several other countries, e.g., a rapid disappearance of the Kappa variant and regional dominance by the Delta variant due to higher infection and transmissibility rates.[15]

Based on travel history reports and phylogenetic analysis, we found that the presence of BA.2.40 in West Kalimantan was primarily due to travelers from Sarawak (Malaysia). Many countries are at risk of COVID-19 due to the effect of internationally imported cases, e.g., Hong Kong and Taiwan.[16] Shandong province (China) was also at high risk of COVID-19 from imported cases due to the peak of the pandemic in South Korea and Japan.[17] However, in contrast, another study reported that relaxation of border control measures for inbound travelers from low-risk countries did not pose a higher risk of COVID-19 outbreak than tighter controls for high-risk countries.[18]

Nucleotide mutation is the survival mechanism of coronavirus to evade the host's immune system.[19] The first reported mutation (D614G) that rapidly spread across the world was identified in February 2020.[20] D614G mutation enhances viral load in the upper respiratory tract, replicates lung epithelial cells, lowers RT-PCR cycle thresholds, and increases interhuman transmission. This discovery was alarming, given that possible future mutations could lead to a more difficult situation.[21-24] Genomic surveillance of SARS-CoV-2 allows optimum contact tracing, as well as tracing the transcontinental origin and history of the virus. During the past two years, the WHO has defined at least 12 variants, namely Alpha, Beta, Gamma, Delta, Epsilon, Zeta, Eta, Theta, Iota, Kappa, Lambda, and Omicron.[25] Omicron is the latest VOC reported in late November 2021 and classified as B.1.1.529, BA.1, BA.2, BA.3, BA.4, BA.5, and descendent sublineages.[1]

The main impact of SARS-CoV-2 mutations is related to mutation of the spike protein, responsible for viral binding to the host cell.[26] Spike protein has a crown-like appearance on the surface of the SARS-CoV-2 virus, allowing it to interact with host cells by binding to receptor angiotensin-converting enzyme 2 (ACE2).[26] Spike protein consists of two subunits, namely subunit 1 (S1) and subunit 2 (S2). S1 binds the virus to the host cell receptors to initiate virus infection. RBD is located in S1 and binds with

cell receptor ACE2.[25] Mutation in this area is crucial for the interaction with receptors and subsequently immune recognition. On the other hand, S2 facilitates viral fusion to the target cell membrane and viral entry.[27] BA.2.40 has 31 sites of mutation on the spike protein, 26 sites of mutation in S1, and five sites in S2. Several mutations of the spike protein have been reported to be associated with host antibodies. Modification of nucleotide barcode in G339D and N440K allows the virus to escape the host-neutralizing antibodies.[28] N440K is reported to be involved in cases of reinfection associated with immune escape and lower vaccine efficiency.[29, 30] These two, together with S373P and S375F mutations, are reported to significantly enhance the interaction between spike protein and ACE2 receptor in the host.[31] Mutations in S371F, D405N, and R408S are reported to reduce the effectiveness of sarbecovirus neutralizing antibodies.[32] Mutation of T19I is associated with mortality,[33] which is specifically found in BA.2 but not in Omicron B.1.617.2, BA.1.1, and BA.1.[27] Together with T19I, K417N and G142D have lower affinity for antibodies.[31] Another interesting mutation has been reported in T376A that reduced the ability of the virus to infect host cells.[34] This means that some variants do not affect the severity of COVID-19.

Several studies reported the impact of spike protein mutations. A previous study showed N501Y and S477N mutations significantly decreased the neutralization activity of some monoclonal antibodies, resulting in immune escape. Furthermore, N501Y and T478K mutations in RBD increased the binding affinity of spike protein for human ACE2.[35] Amino acids, specifically at E484 and N501 sites, increased binding affinity between RBD and ACE2.[36] An *in-silico* study used a method to predict the effect of mutations on spike protein stability. They reported that all amino acid changes caused a decrease in structural stability. Based on functional effect analysis, they showed that E484A, Y505H, N764K, and N969K mutations decrease spike protein's function. Mutation of E484A, located in the RBD, affects viral transmission and Y505H mutation was predicted to negatively affect spike functionality and susceptibility to the disease. On the other hand, mutation of N764K and N969K affected spike functionality, but at the same time exacerbated disease severity.[37] E484A mutation has less affinity for ACE2 than N501Y and was predicted to impact the immunogenicity of RBD protein and decrease the pathogenicity and probability of diseases induced by the protein.[36] A point mutation on Q493R, Q498R, and H655Y decreases spike protein stability but does not disrupt spike functionality and disease severity.[37] Another study reported that the D614G mutation increases the infectivity of the COVID-19 virus.[21]

In the present study, as shown in table 2 and figure 3, we did not find HV69-70 deletion in our samples (nonprobable). This finding is in contrast with other omicron variants that had HV69-70 deletion. BA.2 and its descendants, as well as XBB (another Omicron sublineage), do not have HV69-70 deletion within the spike protein, which is used as a marker to detect an Omicron variant (figure 3). HV69-70 deletion in spike protein allows Omicron variants to be easily identified using the proxy marker of S-gene target failure (SGTF).[38] SGTF has high accuracy in screening for Alpha (98%) and Omicron (100%) variants. It is therefore strongly recommended for early screening of Omicron worldwide. Since January 2022, the Indonesian Ministry of Health has also recommended the SGTF method for early detection of Omicron variants. However, despite its widespread use, this marker should be re-evaluated for screening purposes since our results showed no HV69-70 deletion in the spike protein of BA.2.40.

The main limitation of the study was incomplete clinical data related to travel history reports, limiting our observational analysis. In addition, we could not fully verify that travelers arriving at the international border crossing point had COVID-19.

Conclusion

Omicron lineage BA.2.40 was only detected in West Kalimantan, Indonesia. Phylogenetic analysis showed that BA.2.40 had the same origin as the variant in Malaysia, indicating the effect of internationally imported cases. Genomic analysis of BA.2.40 showed 31 spike mutations in various proteins that differed from other Omicron variants, e.g., Q493R mutation. The use of the SGTF method for early detection of Omicron variants should be re-evaluated since no HV69-70 deletion in the spike protein of BA.2.40 was detected.

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Authors' Contribution

DF.L, A.P: Study concept, data interpretation,

drafting, and revising of the manuscript. M.M: Data analysis and interpretation, drafting and revising of the manuscript. V.N, A.A: Data collection, molecular testing, screening, and sequencing data. All authors have read and approved the final manuscript and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Conflict of Interest: None declared.

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Original Article

Human Papillomavirus-Associated Oral Epithelial Dysplasia: A Practical Approach to Make the Diagnosis

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What's Known

- Human papillomavirus (HPV) plays a significant role in the development of oropharyngeal dysplasia.
- The role of HPV in oral dysplasia has been proven.
- The Chromogenic *in situ* hybridization (CISH) method is standard for determining the presence of HPV in oropharyngeal dysplasia.
- HPV diagnostic method in oral dysplasia needs more studies.

What's New

- HPV has the potential to cause oral dysplasia, as indicated by a prevalence rate of 9.25%.
- HPV has not shown a different incidence in different severity of dysplasia.
- In some cases, polymerase chain reaction (PCR) is necessary in addition to CISH to detect HPV in oral dysplasia.

Abstract

Background: High-risk Human Papillomavirus (HPV) genotypes are found in malignant oral epithelial lesions, and HPV infection is proposed as a risk factor for initiating Squamous cell carcinoma (SCC) in the head and neck region. This study suggests a practical approach to detect HPV in HPV-associated oral epithelial dysplasia (HAOED).

Methods: Fifty-four oral epithelial dysplasia specimens were examined, comprising twenty-seven cases diagnosed with high-grade dysplasia and twenty-seven cases diagnosed with low-grade dysplasia using a binary grading system. To assess the cases for HPV, the specimens were examined for p16 protein using an immunohistochemical (IHC) study, and then, the Chromatin *In Situ* Hybridization (CISH) test was performed for all positive cases. Chromatin Immunoprecipitation-Polymerase Chain Reaction (ChIP-PCR) was performed on CISH-positive specimens to assess the outcome. This cross-sectional study was conducted in 2020 at Tehran University of Medical Science. SPSS software version 22.0 was used to perform the Chi square or Fisher's exact test to examine the relationship between variables (statistically significant level $P<0.05$).

Results: The expression of p16 protein was not associated with the severity of epithelial dysplasia (81.5% in low-grade and 59.2% in high-grade cases) ($P=0.16$). Moreover, according to the CISH test result, 9.25% of all specimens were positive ($P>0.99$), and in the nine cases, undergone the ChIP-PCR study, two cases (22.2%) showed positivity for HPV-16, while one case (11.1%) demonstrated positivity for HPV-51.

Conclusion: Regarding HAOED, here, we proposed a step-by-step combination approach using different diagnostic methods, including IHC for p16 protein, CISH, and ChIP-PCR based on a complementary algorithm.

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Keywords ● Human papillomavirus virus ● Squamous cell carcinoma of head and neck ● Immunohistochemistry ● *In situ* hybridization ● Polymerase chain reaction

Introduction

Human papillomavirus (HPV) is a well-known etiologic factor in the development of uterine cervical cancers,[1] and oropharyngeal carcinomas.[2] Regarding the distinct clinical behavior of

HPV-associated OPCs, pathologists are committed to clarifying the HPV status of this cancer based on the recently published eighth edition of the American Joint Committee on Cancer (AJCC) staging guideline, that HPV-positive cancers are down-staged from IV to I in comparison with the seventh edition.[3] Additionally, although smoking tobacco and alcohol consumption are the main risk factors for initiation of Oral Squamous Cell Carcinoma (OSCC), the role of high-risk HPV infection is recently associated with the pathogenesis of a subset of this cancer as a cofactor[4] through molecular alteration of epigenetic factors.[5]

Oral Epithelial Dysplasia (OED) is a histopathologically challenging topic for a step of epithelial alteration between normal condition and malignancy, and it is now well-described as an Oral Potentially Malignant Disorder (OPMD).[6] Clinical presentation of OED mostly ranges from leukoplakia to erythroplakia or an irregular mix of these lesions.[7] In the past 50 years, many attempts have been made to provide a reproducible OED grading system and practically link pathologists to clinicians for an effective therapeutic intervention without overtreatment.[8]

Meanwhile, from the primary introduction of "Koilocytic dysplasia",[9] several studies have proposed a distinct uncommon subtype of OED as "HPV-associated Oral Epithelial Dysplasia" (HAOED)[10] and have made the diagnosis of this OPMD more critical and complicated. A recently published meta-analysis showed that 25.3 percent of OEDs are associated with HPV infection; though the method of HPV detection- immunohistochemistry, PCR, or *In Situ* hybridization (ISH)- has a significant impact on the sensitivity of the detection.[11] Although high-risk HPVs- including HPV16- are commonly identified in HAOED similar to oropharyngeal cancers, long-term follow-up is necessary to recognize any clinical difference between the malignant transformation of positive and negative cases.[12]

The present study utilized various HPV detection methods in HAOED cases to identify a feasible, sensitive, and reproducible algorithm to differentiate HAOED from common OED cases. Due to the multifactorial nature of oral dysplasia such as SCC and the effect of various factors on it and also due to the proven role of HPV in dysplasia cases and the onset of SCCs in the head and neck region, aimed to evaluate the presence of HPV in OED cases using several diagnostic methods.

Patients and Methods

Patients and Specimens

Formalin-fixed paraffin-embedded blocks of OED cases were obtained from the Oral and Maxillofacial Pathology Department's laboratory, Tehran University of Medical Sciences (TUMS), Tehran, Iran. The inclusion criteria were the diagnosis of OED in their pathologic reports and the absence of inflammation with lichenoid pattern in the target tissue in histopathological examination. In all stages of this study, samples were previously prepared for diagnostic purposes from patients and were not for this study. For this reason, informed consent was not obtained from the patients. This cross-sectional study was conducted in 2020 at Tehran University of Medical Science and ethically approved by the Ethics Committee of the School of Dentistry, Tehran University of Medical Sciences (No. IR.TUMS.DENTISTRY.REC.1399.096).

Demographic information of all the patients, including age, sex, and anatomical site of the lesion, were extracted from the submitted records.

In accordance with the findings of Angerio and others study,[13] the frequency of high-risk HPV was observed to be 8% and 46% in samples with low and high degrees of hyperplasia, respectively. To scrutinize the variance between the two ratios, amounting to 38%, while maintaining a type 1 error rate of 5% and a statistical power of 80%, at least 17 samples are necessary in each group. As a precautionary measure, if the difference in frequency between the groups is postulated to be 30%, at least 27 samples are required in each group. To estimate the required sample size for evaluating the hypothesis of comparing the prevalence of HPV between samples with low and high degrees of hyperplasia, the following statistical formula was employed to compare two proportions.[14]

$$n = \frac{(Z_{1-\alpha/2} + Z_{1-\beta})^2 (p_1(1-p_1) + p_2(1-p_2))}{(p_1 - p_2)^2}$$

Microscopic Evaluation and Grading

Histopathologic assessment of the specimens was performed on hematoxylin and eosin (H&E) stained 4-µm-thick paraffin sections. Microscopic findings, consisting of acanthosis, hyperkeratosis, papillomatosis, verrucous pattern, and koilocyte-like cells, were included in the histopathologic examination.

All OEDs were graded according to the binary grading system[15, 16] and were divided into two groups of ''low-risk'' or ''high-risk'' cases. The binary system was proposed by Omar Kojan and colleagues and divided dysplastic lesions into two groups: high-grade and low-grade. High-grade dysplasia lesions are determined by having at least four criteria of changes in the

general shape of the epithelium and five criteria of cytological changes of the WHO criteria based on microscopic observation. Low-grade dysplasia is determined based on observing less than four general changes in the epithelium or less than five cytological changes (WHO criteria).[15]

Immunohistochemical Study

An immunohistochemical (IHC) study for p16 was performed on 4-µm-thick paraffin sections. All the sections were deparaffinized and rehydrated. Primary antibody (Mouse anti-human p16INK4A, Monoclonal Antibody, Clone MX007, Master Diagnostica, Spain; dilution 1:40, pH 7.3) incubated for 10 min. Master Polymer Plus Detection System (HRP) (DAB included; Master Diagnostica-000237QK, Spain) was used as the detection system. Finally, the slides were counterstained with hematoxylin and mounted. HPV-positive oropharyngeal carcinoma specimen was used as a positive control. Omitting the antibody and using phosphate-buffered saline were performed as a negative control.

Two experienced oral pathologists (S.D. and P.A.) blindly assessed stained slides for p16 expression. Both nuclear and combined nuclear/cytoplasmic stained specimens were considered positive. The positive cells were scored according to the proportion of stained cells as follows: 0%=0, 1-10%=1, 11-50%=2, 51-80%=3, 81-100%=4. It finally scored as follows:

Score 0: Negative (no positive cells)

Score 1: Focally positive (1-80% of cells show positivity).

Score 2: Diffusely positive (81-100% of cells show positivity).[17]

Chromogenic In Situ Hybridization

A Chromogenic In Situ Hybridization (CISH) study was performed in 37 cases, showing scores of 1 and 2 in the IHC study for p16. Briefly, after dehydration in 100% ethanol for 1 min, 10 µl of ZytoFast CISH Probe (ZytoFast PLUS CISH Implementation Kit AP-Permanent Red, Zytovision, Prod. No. T-1151-40, Germany) for HPV genotypes 16, 18, 31, and 33 (ZytoFast HPV type 16/18 Probe, Prod. No. T-1056-400, Germany and ZytoFast HPV type 31/33 Probe, Prod. No. T-1057-400, Germany) were used, and the slides were assessed using light microscopy.

Chromatin Immunoprecipitation PCR

Among the samples that were subjected to the IHC test for p16 protein and CISH, to finalize and confirm the diagnosis of the presence or absence of HPV, the HPV Direct Flow CHIP test was performed on two groups of samples, including samples whose result was focally positive in the p16 test and equivocal in the CISH test (N=5), and samples whose results were diffusely positive in the p16 test and negative in the CISH test (N=4). We used the HPV Direct-Flow Chip Kit (HS12, PCR Reagents, Master Diagnostia, Granada, Spain). In this protocol, the clinical samples can be amplified directly with no need to extract DNA. Amplification cycling conditions in the peqSTAR XS ThermoCycler followed the manufacturer's instructions. 5 µL of the liquid suspension under the paraffin layer was used as a DNA template. Automated reverse hybridization was performed on hybriSpot 24 (HS24, ref.MAD-003930MU-HS24, Master Diagnostica, Spain), which allows the DNA target molecules to cross the membrane and bind to the complementary probes. NBT-BCIP substrates were added to colorimetric detection by detecting alkaline phosphatase activity and creating insoluble purple precipitates.

Statistical Analysis

The Chi square test (or Fisher's exact test, if appropriate) was performed to investigate the relationship between independent (OED grade and histopathologic factors) and qualitative dependent (IHC for p16 and CISH test for high-risk HPV) variables. The statistically significant level was considered less than 0.05. For quantitative variables, because they did not follow the normal distribution, the median was reported along with the first and third quartiles (Q1 and Q3). Statistical analyses were performed using SPSS software version 22.0 (IBM, Armonk, NY, USA).

Results

A total of fifty-four OED cases were examined, comprising twenty-seven cases diagnosed with high-grade dysplasia and twenty-seven cases diagnosed with low-grade dysplasia. In the low-grade group, 12 females (44.4%) and 15 males (55.6%), and in the high-grade group, 17 females (63.0%) and 10 males (37.0%) were included. The mean age of the patients with low-grade and high-grade dysplasia was 56.93 (ranging from 29 to 78) and 62.89 (ranging from 29 to 78), respectively.

Immunohistochemistry

The percentage of p16 protein expression in both low-grade and high-grade groups indicates a median of 10%. Besides, in the low-grade group, the inter-quarter range was 35% (Q1=5, Q3=40), and in the high-grade group, the inter-quarter range was 50% (Q1=0, Q3=50).

According to the p16 protein expression, in the low-grade group, five out of 27 cases (18.5%) showed a final score of 0, 20 out of 27 cases (74.1%) demonstrated a final score of 1, and two out of 27 cases (7.4 %) were observed with a final score of 2. Furthermore, in the high-grade group, 11 out of 27 specimens (40.7%) showed a final score of 0, 13 out of 27 specimens (48.1%) demonstrated a final score of 1, and three out of 27 specimens (11.1%) were observed with a final score of 2 (figure 1).

There was no significant association between the expression of p16 protein expression and the severity of dysplasia (P=0.16).

Chromogenic In Situ Hybridization

The CISH study for HPV-16 and -18 showed three positive cases (11.1%) in the low-grade group and two positive cases (7.4%) in the high-grade group. There was no significant association between HPV-16 and -18 positivity and the severity of dysplasia (P>0.99).

Moreover, the CISH study for HPV-31 and -33 demonstrated one positive case (3.7%) in the low-grade group and two positive cases (7.4%) in the high-grade group, which showed no significant association between HPV-31 and -33 positivity and the severity of dysplasia (P>0.99).

Furthermore, three specimens showed positive expression for all HPV-16, -18, -31, and -33 using a CISH study (figure 2).

Chromatin Immunoprecipitation PCR

Out of the five samples whose IHC staining result was diffusely positive and the CISH test result was negative, in the ChIP-PCR test, four samples were negative for the presence of HR-HPV, and only one sample reported the presence of HPV-51.

Out of the four samples, the final score of IHC staining was focally positive, and in the CISH tests the results were equivocal. In the ChIP-PCR test for the presence of HR-HPV, HPV-16 was positive in two samples, and in two samples, the presence of the virus was reported as negative.

In total, nine specimens were assessed for high-risk HPVs using ChIP-PCR, and six cases (77.7%) were negative, two cases (22.2%) were positive for HPV-16, and one case (11.1%) was positive for HPV-51.

Finally, three cases from the low-grade group and three cases from the high-grade group showed positivity, and there was no significant association between the presence of high-risk

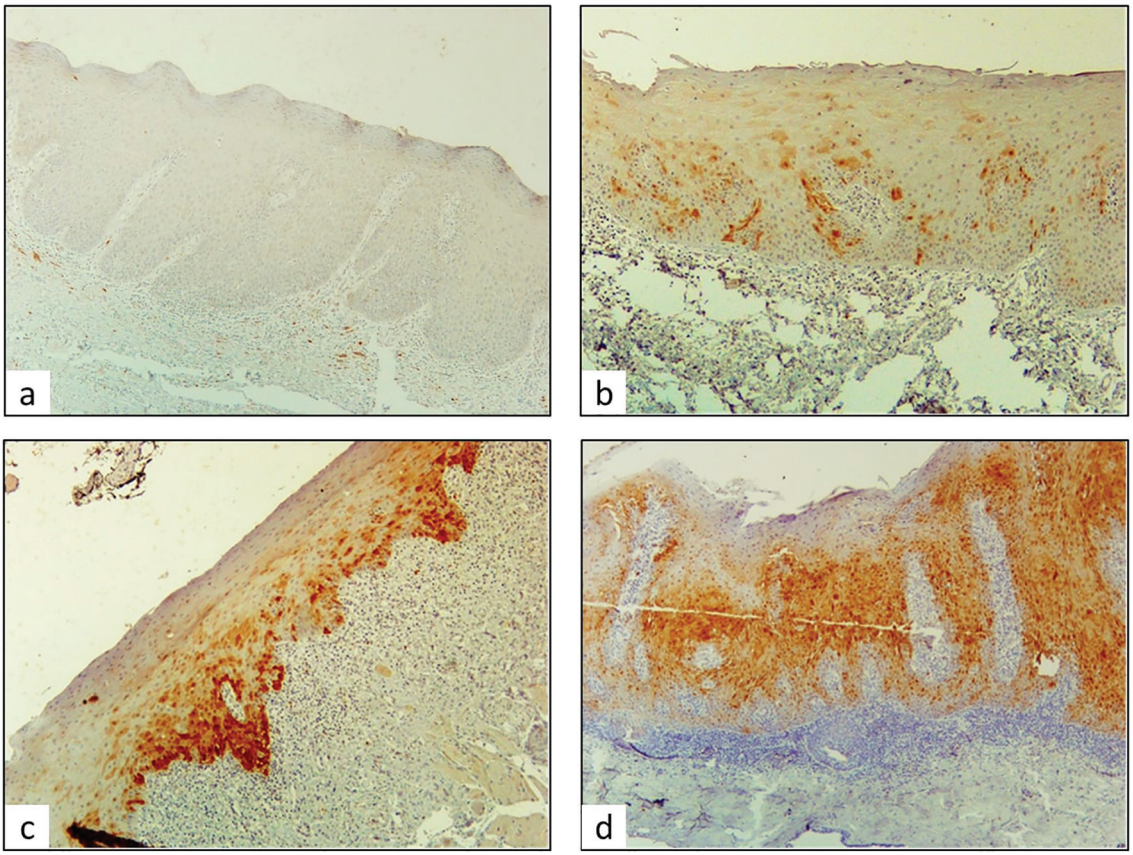

Figure 1: The Immunohistochemistry (IHC) study for p16 was performed for all samples, the brown stained cells indicate p16 expression. a) A specimen in which no p16 protein expression was observed (×40). b) A specimen in which 30% p16 protein expression was observed (×100). c) A specimen in which 60% p16 protein expression was observed (×100). d) A specimen in which 80% p16 protein expression was observed (×40).

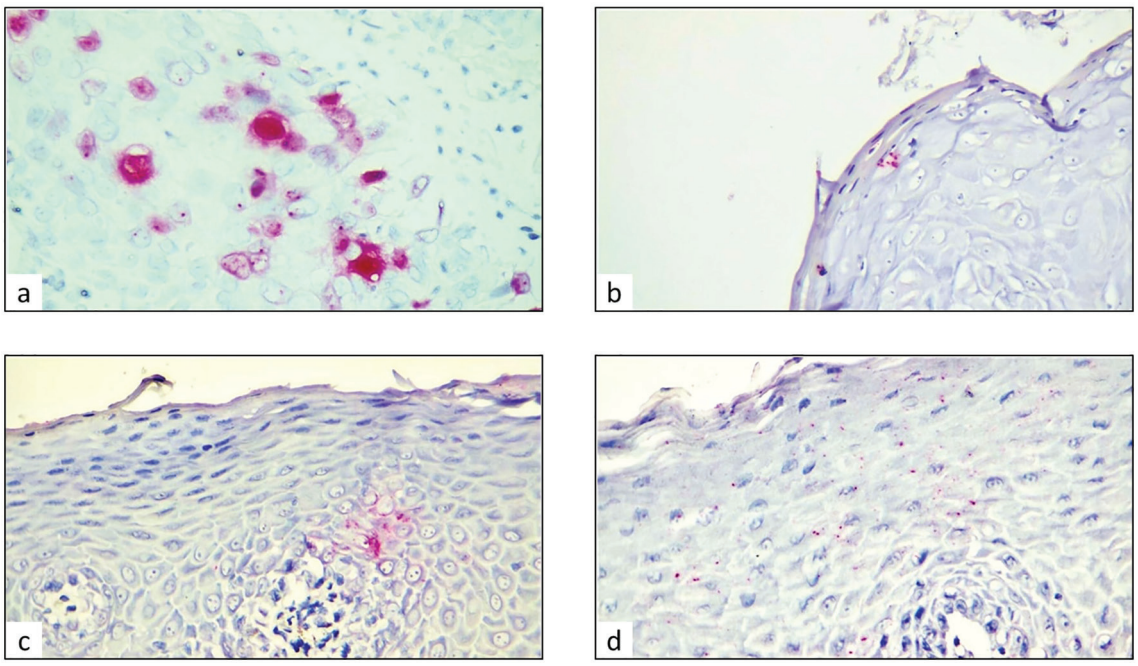

Figure 2: Chromogenic in situ hybridization (CISH) study for human papillomavirus (HPV) was performed for the samples with scores 1 and 2 in the Immunohistochemistry (IHC) study for p16. a) A specimen of uterine cervical tissue, which was performed for a positive control for the CISH study (×400). b, c, d) Specimens that were positive in the CISH study (×400).

Table 1: Microscopic findings in low-grade and high-grade dysplasia cases

Variables		Low-grade dysplasia N=27 N (%)	High-grade dysplasia N=27 N (%)	Odds ratio (95% confidence interval)	P value*
Papillomatosis	Present	8 (29.6%)	13 (48.1%)	2.21 (0.72-6.75)	0.16
	Absent	19 (70.4%)	14 (51.9%)		
Verrucous	Present	3 (11.1%)	2 (7.4%)	0.64 (0.10-4.17)	>0.99
	Absent	24 (88.9%)	25 (92.4%)		
Hyperkeratosis	Present	16 (59.2%)	13 (48.1%)	0.64 (0.22-1.87)	0.41
	Absent	11 (40.8%)	14 (51.9%)		
Acanthosis	Present	26 (96.3%)	26 (96.3%)	1.00 (0.06-16.85)	>0.99
	Absent	1 (3.7%)	1 (3.7%)		
Koilocyte	Present	9 (33.3%)	4 (14.8%)	0.35 (0.09-1.31)	0.11
	Absent	18 (66.7%)	23 (85.2%)		

*A statistical analysis using either the Chi square test or Fisher's exact test, as deemed appropriate, was conducted.

Table 2: Microscopic findings in human papillomavirus-positive and -negative cases

Variables		HPV-Positive N=6 N (%)	HPV-Negative N=48 N (%)	Odds ratio (95% confidence interval)	P value*
Papillomatosis	Positive	3 (50%)	18 (37.5%)	1.67 (0.03-9-16)	0.67
	Negative	3 (50%)	30 (62.5%)		
Verrucous	Positive	2 (33.3%)	3 (6.2%)	7.50 (0.95-59.89)	0.09
	Negative	4 (66.7%)	45 (93.8%)		
Hyperkeratosis	Positive	5 (83.3%)	24 (50%)	5.00 (0.54-46.05)	0.20
	Negative	1 (16.7%)	24 (50%)		
Acanthosis	Positive	6 (100%)	46 (95.8%)	-	>0.99
	Negative	0 (0%)	2 (4.2%)		
Koilocyte	Positive	2 (33.3%)	11 (22.9%)	1.68 (0.27-10.44)	0.62
	Negative	4 (66.7%)	37 (77.1%)		

*A statistical analysis using either the Chi square test or Fisher's exact test, as deemed appropriate, was conducted.

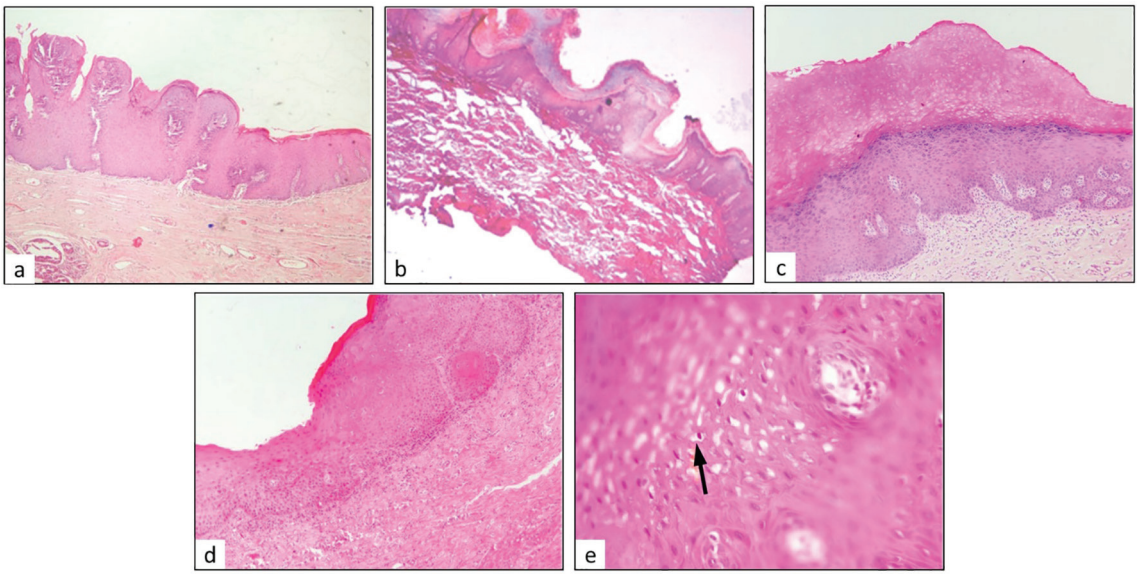

Figure 3: Histopathological features were observed and analyzed in all samples (H&E staining). a) Epithelial tissue with prominent papillomatosis was observed (×40). b) Epithelial tissue with verrucous features was observed (×40). c) Epithelial tissue with hyperkeratosis was observed (×40). d) Epithelial tissue with acanthosis was observed (×100). e) Epithelial tissue with koilocytic cells was observed (×400).

HPVs based on all IHC, CISH, and ChIP-PCR studies and the severity of dysplasia (P>0.99).

Microscopic Evaluation

Microscopic findings including papillomatosis, verrucous, hyperkeratosis, acanthosis, and the presence of koilocyte-like cells are shown in tables 1 and 2 (figure 3).

Discussion

The results of this study showed that the expression of p16 protein was not associated with the severity of epithelial dysplasia (81.5% in low-grade and 59.2% in high-grade cases). Additionally, according to the CISH test result, 9.25% of all specimens were positive, and in the nine cases undergone the ChIP-PCR study, two cases (22.2%) showed positivity for HPV-16, and one case (11.1%) demonstrated positivity for HPV-51.

For clinicians, OED is a dilemma because of the unknown potential transformation risk to OSCC. OED is a range of tissue and cellular

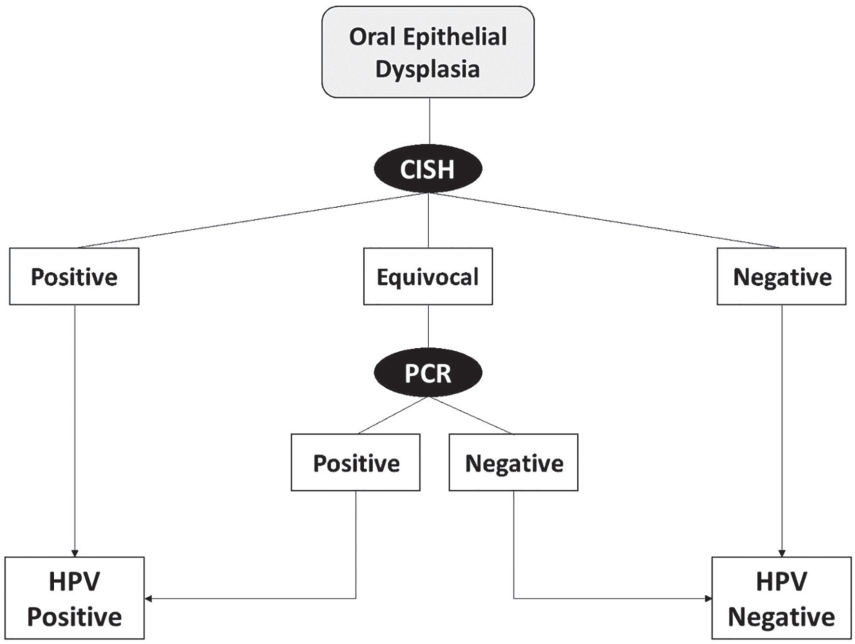

Figure 4: The proposed algorithm to detect human papillomavirus (HPV) in HPV-associated oral epithelial dysplasia (HAOED). (CISH: Chromogenic in situ hybridization, PCR: Polymerase chain reaction, HPV: Human papillomavirus)

changes restricted to the surface epithelial layers without any invasion into the underlying connective tissue.[18]

Although these alterations commonly manifest all the time,[19] HPV has been established as a primary cause of squamous cell carcinoma of the uterine cervix and oropharyngeal region.[20-22] There is no definitive evidence of the carcinogenic role of HPVs in the oral cavity[23] and an extremely variable range of HPV detection, from 0 to 100 percent, is reported in malignant and potentially malignant oral lesions.[24]

One of the most important reasons for this variability is the different methods of HPV detection. Furthermore, some of them are associated with the lesion's anatomical site, based on viral integration into the cellular genome.[25] Another noticeable point that may affect HPV detection is the different grades of dysplasia, especially when different grading systems are used. For example, we analyzed HPV detection in 54 cases of OED and graded them using the binary system in addition to IHC assessment of p16 protein, CISH, and PCR methods. In our study, 70.4% of the specimens demonstrated positive immunoreaction for the p16 antibody.

Although no significant difference between different dysplasia grades was found in our study, some studies demonstrated different expressions of p16 in different grades of dysplasia.[24, 25]

Besides, there are controversial studies about histopathologic features associated with HPV-positive OED.[12] These histopathologic features are named virus-associated dysplasia/bowenoid papulosis.[26]

Woo and others reported specific histopathologic features in 100% of the cases with positive p16 and high-risk HPV-DNA.[27] However, some studies did not confirm the association between some specific histopathologic features and the expression of p16 or HPV-DNA,[25, 28] similar to our study.

A few studies reported an association between HPV and OED, which was confirmed by both positive p16 findings and DNA ISH.[12, 25, 26]

As mentioned, there are various methods for HPV detection, and choosing the best method with the highest sensitivity seems a big dilemma.

Jayaprakash and others, in a meta-analysis on OED, reported that PCR shows significantly higher sensitivity than ISH for the evaluation of HPV-16 and -18.[11]

In another review in 2017, it was reported that although ISH study carries a low sensitivity, PCR has a risk of false positive results in the detection of HPV, especially endemic infections, which are not commonly associated with pathologic tumors.[29]

In our study, we used the CISH method for all p16 positive cases and the PCR method to confirm negative-CISH and diffusely p16 positive cases and equivocal CISH results with focally positive p16 cases.

We showed that the expression of p16 protein could not be a reliable indicator of HPV in OED, regarding all five diffusely-p16 positive cases, which showed negative results using both CISH and ChIP-PCR studies.

The results are consistent with previous studies on HPV DNA integration into the host genome in potentially malignant oral lesions.[24, 25] Although the IHC study for p16 is available and feasible, it is not a sensitive method to detect HPV in OED. However, unlike OED, there is a strong association between HPV and the expression of p16 protein in oropharyngeal squamous cell carcinoma.[29]

In the absence of a universal standardized method to detect HPV in HAOED, we proposed a step-by-step combination approach using different diagnostic methods based on a complementary algorithm (figure 4).

To determine a protocol for checking the presence of high-risk HPV subtypes in oral dysplastic tissues using existing diagnostic methods, the p16 method for HPV detection and subtype determination in oral dysplasia samples is not definitively helpful. It is better to use the CISH diagnostic method from the very beginning, which can identify the high-risk subtypes of HPV. Besides, for samples that have been paraffinized, this diagnostic method has shown high accuracy. In the samples whose CISH test results are reported positive and the staining pattern is similar to the positive control, the presence of HPV in them should be considered positive. In the samples whose result of the CISH test is reported as negative and the staining pattern is similar to the positive control, the presence of HPV in them should be considered definitively positive. In some dyed samples, the percentage of staining may be seen at a deficient level compared to the positive control, which can be used to diagnose these samples by PCR test definitively. In the PCR method, it is possible to distinguish the types of HPV, and good accuracy clearly has been shown for this method in frozen and freshly biopsied tissues in studies. However, for paraffined samples, it is better as an auxiliary method for CISH testing.

All HPV detection methods have their advantages and disadvantages considering cost, sensitivity, DNA or protein detection, and others. Here, we recommended an algorithm to

collect the benefits of all detection methods in a well-designed order. It is clear that using any single method of HPV detection has significant limitations.[30]

Among the limitations of the present study, the following can be mentioned: the difficulty in performing laboratory procedures, the sensitivity of diagnostic tests to the technique of use, the need for a skilled technician to perform these tests, and the small volume of tissues, since some of the initial samples are biopsies.

Conclusion

At the current state of knowledge, HPV detection methods for HAOED still remain a controversial issue. It is very important to have a practical combination of methods to accurately detect HPV in OED lesions. A simple step-by-step algorithm to facilitate the diagnosis of HAOED is suggested here.

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Authors' Contribution

K.P: Specimen collection and experimentation, drafting; S.D: Study design, evaluation of microscopic slides stained with IHC, and drafting; H.S: Analyzing CISH and ChIP-PCR results, reviewing the manuscript; P.A: Study design, evaluation of microscopic slides stained with IHC, and reviewing the manuscript; AR.Sh: statistical data analysis and reviewing the manuscript, S.A: Specimen collection and experimentation, and reviewing the manuscript. All authors have read and approved the final manuscript and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Conflict of Interest: None declared.

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Brief Report

The Potential Role of Autophagy in Progression of Liver Fibrosis in Chronic Hepatitis B Patients Receiving Antiviral Treatment: A Brief Report

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Abstract

Despite antiviral treatment, some patients with chronic hepatitis B (CHB) progress to cirrhosis. Enhancement of autophagy was implicated in the proliferation of hepatitis B in hepatocytes. This study aimed to evaluate the potential role of autophagy in the progression of liver fibrosis in patients receiving antiviral treatments and having completely inhibited viral replication. This descriptive-analytical study was designed and conducted in 2020 at Mottahhari Hepatitis Clinic affiliated with Shiraz University of Medical Science (Shiraz, Iran). Patients who were on anti-hepatitis B nucleotide treatments for at least two years, and those who were not cirrhotic at baseline but later progressed to cirrhosis were identified to be included in the case group. Besides, for the control group, patients on the nucleotide regimens who did not have cirrhosis at baseline or during follow-up were randomly selected. Ultimately, 16 cases and 14 controls were included in the study. Data were analyzed using SPSS software, and $P<0.05$ was considered statistically significant. Serum Beclin-1 and LC3 levels were compared between the two groups using enzyme-linked immunosorbent assays. The *t* test was used to assess the statistical differences between the case and control groups. Beclin-1 level was significantly higher in cirrhosis patients than the control group (1283±244 vs. 1063±257, P=0.024). However, there was no statistical difference between the level of LC3 in the cirrhotic group (168±31) and the control group (150±16) (P=0.065). Autophagy may have a role in the progression of cirrhosis in patients with CHB. Future larger prospective studies are required to determine the effect of blocking on the progression of liver disease in this population.

A preprint of this study was published at https://www.researchsquare.com/article/rs-1435490/v1.pdf.

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Keywords ● Autophagy ● Hepatitis B, chronic ● Fibrosis ● Beclin-1

What's Known

- The hepatitis C virus uses cellular autophagy in viral replication.
- Chloroquine was used as an autophagy suppressor.

What's New

- Cirrhosis emerged unexpectedly in a small subset of chronic hepatitis B patients treated with HBV DNA-negative nucleotide analogues. They had higher levels of Beclin-1 (an autophagy marker), suggesting that viral suppression is insufficient in liver disease and autophagy could be a possible mechanism.

Introduction

Hepatitis B virus (HBV) is a double-stranded DNA virus, which is one of the leading causes of hepatocellular carcinoma and liver cirrhosis in the world.[1] It is estimated that about 255 million people have chronic HBV infection worldwide.[2] The risk of liver cirrhosis

is higher in individuals with higher serum levels of HBV DNA. Moreover, suppressing viral replication was reported to reduce the risk of cirrhosis progression and hepatocellular carcinoma (HCC).[3]

Other risk factors, including co-infection with the hepatitis D virus, hepatitis B e-antigen positive, concomitant diabetes mellitus, and old age, increased the risk of progression to cirrhosis.[4] In somatic cells, autophagy is the primary degradation system. Eukaryotic autophagy involves several mechanisms to degenerate the short-lived protein.[5] The pathogenesis and progression of HCC are associated with decreased autophagy.[6]

A previous study reported numerous cases of chronic HBV infection-inducing autophagy.[7] This activation might be more prominent in HCC patients with chronic HBV infection.[8] Therefore, developing treatments that target autophagy might have a role in delaying or even retreating the liver injury induced by HBV, as well as fatty liver disease and HCC.[6, 9] Blockade of autophagy-mediated processes could provide new opportunities for preventing or reversing cirrhosis.

Beclin-1, a key autophagic gene, was found to be overexpressed in various human malignancies.[6] Furthermore, LC3-II was specifically associated with autophagosomes and autolysosomes.[10]

Beclin-1 is a crucial autophagic agent. The recruitment and activation of Beclin-1 are some of the initial steps in the construction of autophagosomes from pre-autophagic structures.[11] Beclin-1 acts as a tumor suppressor and is an essential autophagy mediator. Beclin-1 also interacts with Bcl-2 and can induce apoptosis.[9]

This study was designed to investigate the potential role of autophagy in the progression of liver fibrosis in chronic hepatitis B (CHB) patients who received antiviral treatment with complete viral replication suppression.

Patients and Methods

This descriptive-analytical study was designed and conducted in 2020 at Mottahhari Hepatitis Clinic, affiliated with Shiraz University of Medical Sciences (Shiraz, Iran). The present study had no selection or sampling procedure, because the study was conducted on all eligible patients who fulfilled the inclusion criteria and volunteered to participate. The inclusion criteria include being between the ages of 18 and 80 years, having at least two years of standard antiviral HBV treatment in the past, and having confirmed reduced viral load in the follow-up studies prior to the diagnosis of cirrhosis. In follow-up, cirrhosis was diagnosed using clinical examination, imaging, including transient elastography, and biochemical markers of fibrosis. The patients with concomitant comorbid diseases such as diabetes mellitus, those who used alcohol at any amount, those with HDV or HCV co-infections, hemochromatosis, and those with a BMI more than 30 at baseline or during follow-up were excluded.

For the control group, we randomly selected from the electronic health records those with the same characteristics but no evidence of cirrhosis in follow-up. The study was approved by the Ethics Committee of Shiraz University of Medical Sciences, Shiraz, Iran (code: IR.SUMS.MED.REC.1399.542). The participants were informed about the goals of the research, and written informed consent was obtained from all the participants. All the participants of the case group (n=16) were not cirrhotic at baseline, and the development of cirrhosis could not have been caused by any other pathologic condition or disease. The control group (n=14) was non-cirrhotic CHB individuals who were referred to Motahhari Hepatitis Clinic, Shiraz University of Medical Sciences, within the same period of time.

Blood samples (10 cc) were obtained from the patients by a venous puncture at the Hepatitis Clinic and centrifuged to be prepared. The separated serum was stored in a freezer at -20 °C until the analysis procedure. Serum Beclin-1 and LC3 levels were measured using an enzyme-linked immune-sorbent assay (Sunlong Biotech Co. Ltd, China) in the Research Laboratory of Shiraz University of Medical Sciences, School of Biochemistry (Shiraz, Iran). The serum assessment process was based on an intra-assay coefficient of variation (CV) of less than 10%, an inter-assay CV of less than 12%, and a lower detection limit of 1 pg/mL.

Statistical Analysis

All the data were analyzed using the SPSS software, version 21 (IBM Statistics, Chicago, USA). Data were expressed as mean±SD. The *t* test was used to compare the statistical differences between the case and control groups. Besides, covariance analysis was performed on age, length of the treatment, and transaminase levels. $P<0.05$ was considered statistically significant.

Results

All Cases admitted in this study were patients with CHB who received viral hepatitis B treatment for at least two years. Moreover, HBV DNA was negative in all cases.

Table 1: Comparison of demographic and biochemical factors between cirrhotic and non-cirrhotic chronic hepatitis B infected patients

Variable	Cirrhotic group (mean±SD)	Non-cirrhotic patients (mean ±SD)	P value*	P value**
Age (years)	60.9±8.5	49.1±10	0.002	-
Treatment duration (years)	11.3±4.8	10±4.2	0.446	-
AST (IU/L)	41.2±25.4	26.6±7.9	0.049	-
ALT (IU/L)	39.4±17.3	31.4±16.2	0.205	-
Plt (×1000/μL)	149±48	215±56	0.002	-
Hb (g/dL)	14.4±1.4	14.2±1.7	0.679	-
AFP (ng/mL)	4.6±1.8	2.4±1.9	0.005	-
Beclin level (pg/mL)	1283±244	1063±257	0.024	0.008
LC3 level (pg/mL)	168±31	150±16	0.065	0.290

*Based on independent *t* test; **Analysis of covariance adjusted by, age, and length of treatment; AST: Aspartate aminotransferase level of plasma; ALT: Alanine aminotransferase level of plasma; Plt: Platelet count; Hb: Hemoglobin; AFP: Alpha Fetoprotein level of plasma; P<0.05 was considered statistically significant.

In total, 30 patients participated in this study. Cirrhosis was diagnosed based on clinical and laboratory findings, as well as transient elastography. Despite treatment and satisfactory virologic response, 16 cases (53.3%) progressed to cirrhosis. The remaining 14 cases (46.6%) were not cirrhotic. The characteristics of the two groups are presented in table 1.

The mean age of cirrhotic and non-cirrhotic patients was 60.9±8.5 and 49.1±10, respectively. The mean age of the cirrhotic group was significantly higher than the non-cirrhotic group (P=0.002). The mean duration of antiviral treatment in the cirrhotic group (11 years) was not statistically different from the non-cirrhotic group (10 years).

Beclin concentration was higher in the cirrhotic patient group than the control group, which was statistically significant (P=0.024). Although the cirrhotic patient group had higher plasma concentrations of LC3, the difference was not statistically significant (P=0.065).

Treatment duration and age were supposed to be confounding variables. However, after adjusting for age, treatment duration, and level of serum transaminases levels using covariance analysis, the mean plasma concentration of Beclin was still significantly higher in the cirrhotic patients (P=0.008). As indicated in table 1, the covariance analysis revealed that mean LC3 concentration was not significantly different in both groups (P=0.290).

Discussion

The findings of the present study indicated that in this series of patients with CHB receiving long-term oral nucleotide analogues, who had negative HBV DNA as well as those who progressed to cirrhosis, had higher levels of Beclin-1. Moreover, the length of treatment and age were supposed to be the confounding variables. Thus, covariance analysis was used to account for them. However, the cirrhotic patients had significantly higher mean plasma concentrations of Beclin.

Despite antiviral treatment, the progression to cirrhosis in these patients might suggest that viral suppression with nucleotide analogues is insufficient for cirrhosis prevention. One of the potential mechanisms causing cirrhosis is autophagy. Beclin-1, one of the markers of early-phase autophagy, was higher in these patients, which could provide proof for this hypothesis.

HBV was found to utilize autophagy as a mechanism for cell proliferation.[12] Despite viral suppression, this mechanism might still be active and cause fibrosis and cirrhosis in some patients. Moreover, other etiologies for the development of cirrhosis was associated with autophagy. It could be a double-edged sword, enhancing pathogens and abnormal cell proliferation, such as malignancy. Therefore, it was implicated that autophagy contributes to the proliferation of HBV.[11]

Autophagy is involved in both the innate and adaptive immune responses to viral hepatitis, such as HBV.[8] Focusing on autophagy may introduce a new opportunity to prevent and even reverse fibrosis in patients with chronic liver disease from any cause, particularly CHB and CHC. Previous studies indicated that the use of chloroquine as a suppressor of autophagy inhibited HCV replication.[13, 14] However, the present study found that patients who developed cirrhosis after receiving antiviral medications had higher serum levels of Beclin-1, a marker of autophagy.

The present study had several limitations. Although none of the patients in these series had cirrhosis based on clinical, endoscopic, imaging findings as well as liver biopsy results, which were available at the time of initiation of anti-viral treatment, some might have had subclinical cirrhosis from the beginning. To address this

issue, a prospective study is required. Tissue studies may reveal further details about the events at the molecular level. Another limitation of our study was the small sample size of our series, because it was limited to a single center. Furthermore, one may argue that it is the effect of fibrosis rather than its cause.

Conclusion

The findings demonstrated that, at least in some patients with CHB, autophagy may play a role in cirrhosis progression despite adequate viral suppression. Moreover, even after controlling for confounding variables such as treatment duration and age, Beclin-1 concentration was significantly higher in the cirrhotic patient group than the control group. Therefore, Beclin-1 and other autophagy mediators might lead to new promising therapies to prevent or even reverse HBV-related liver fibrosis.

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Authors' Contribution

K.BL: Study concept, study design, and drafting; A.S: Performing the study, data management, and drafting; MR.F: Data gathering and data analysis, and critical revision; S.A: Laboratory data management and drafting; P.M: Laboratory data management and drafting; All authors have read and approved the final manuscript and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Conflict of Interest: None declared.

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Letter to Editor

Mesenteric Panniculitis: An Enigma

Dear Editor

Mesenteric panniculitis (MP) is one of the few rare chronic inflammatory disorders with an unknown etiology. This could be due to an autoimmune disease, trauma, ischemia, medications, or allergies. The disease is more common in men and affects all age groups, with a peak prevalence in the sixth and seventh decades of life.[1]

MP is difficult to diagnose due to nonspecific and varying types of clinical presentation. The diagnostic difficulty in this case is further increased by the presence of concurrent renal stones.

On 15 September 2022, a 52-year-old woman from rural North India with a body mass index (BMI) of 25.4 was referred to our hospital. She was admitted with pain in the left side of her abdomen that was insidious in onset, dull hurting, continuous, mild to moderate, and without any radiation. There were no signs of fever, diarrhea, and vomiting. Before being admitted to our hospital, she was referred elsewhere for ultrasonography of the whole abdomen, which revealed a 4 mm renal stone with no other remarkable finding in the rest of the abdomen. Although she was treated for renal stone, her symptoms did not resolve completely. Within 3 months following the initial symptom, a mass developed on the left side of her abdomen, for which she was treated with a repeated course of antibiotics with little result. Nausea, vomiting, dizziness, abdominal distention, and constipation were all symptoms of the mass. However, no fever or diarrhea was reported. To conduct further investigation, written informed consent was obtained from the patient. Contrast-enhanced computed Tomography (CECT) of the whole abdomen was performed. The findings revealed non-obstructive left renal calculi measuring 5.6 mm in size (figure 1) as well as enhancing circumferential mural thickening of the rectum, sigmoid colon, and distal segment of descending colon with perifocal fat stranding and multiple sub centimetric discrete mesenteric lymph nodes with pseudo capsule (figure 2).

Following sigmoid-colonoscopy and biopsy, it was discovered that the rectosigmoid was non-negotiably narrowed with normal mucosa and vascular pattern. The findings of the biopsy pointed to a nonspecific inflammatory pathology with no granulomas or atypical cells. Based on the CECT findings, a provisional diagnosis of MP was determined. Except for total leucocyte counts, which were 14.2×10^9/L and predominantly neutrophils, all the laboratory parameters were within normal ranges.

Methylprednisolone (Pfizer, India), 40 mg per day, was prescribed for 14 days. Then, decreased over the next 14 days while also using a stool softener. After four weeks of medication, her symptoms improved, and her laboratory results became normal. Monthly follow-up abdominal CT scans revealed a remarkable improvement over time (figures 3A and B).

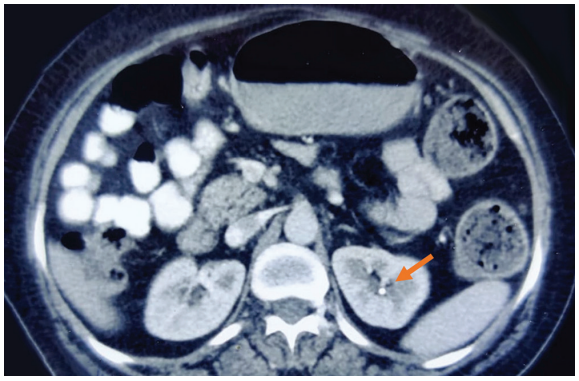

Figure 1: Contrast-enhanced computed tomography of the abdomen shows a left renal stone (size: 5.6 mm).

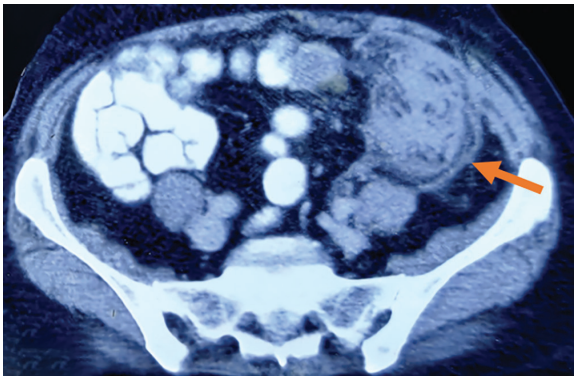

Figure 2: Contrast-enhanced computed tomography of the abdomen shows extensive fat stranding in the descending colon and proximal sigmoid colon suggestive of a large inflammatory mass (orange arrow).

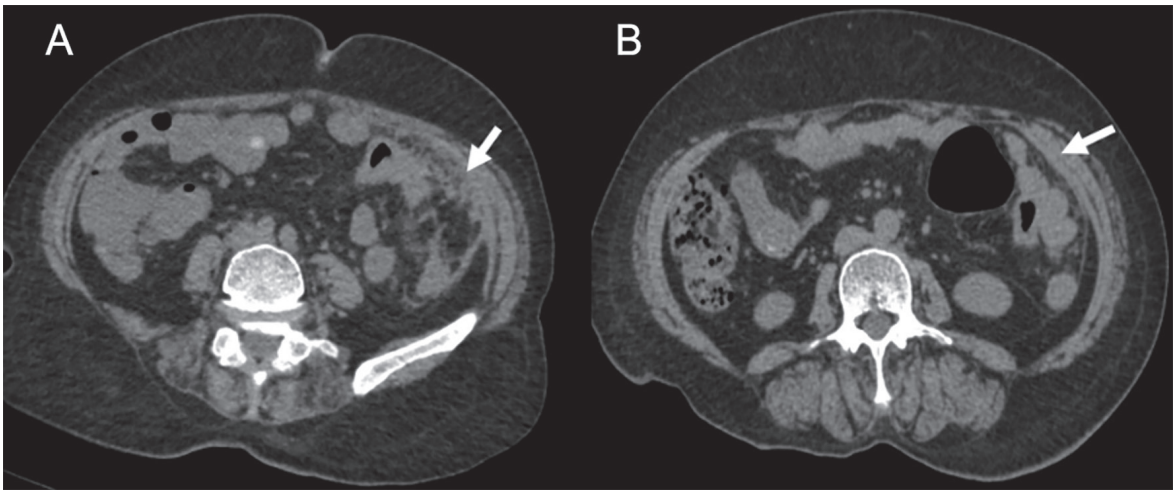

Figure 3: Contrast-enhanced computed tomography of the abdomen shows decreases in the size of inflammatory mass after one month (A), and complete disappearance of mass after three months (B).

Clinical manifestations of MP might range from asymptomatic to symptomatic. MP might be diagnosed as an incidental finding in as much as 7% of cases.[2] Gastrointestinal disturbance, abdominal pain, nausea, vomiting, diarrhea, constipation, weight loss, pyrexia of unknown origin, and chylous ascites are all possible symptoms.[1] The clinical presentation might resemble Crohn's disease.[3] Moreover, it has an association with type 2 diabetes mellitus.[4] Although MP might have an association with several neoplasms, no direct association was found between MP and malignancy.

CECT, magnetic resonance imaging (MRI), and biopsy are among the available diagnostic tools. In CECT, the fat ring sign, and the pseudo capsule sign are the two primary radiological signs, which are considered distinctive features of MP. Even so, the disease is not ruled out if these symptoms are absent. The retractile type of MP might differ significantly from the classic version. Depending on the degree of fibrosis, the retractile form may manifest as a mass that is mainly solid and fibrotic component or as a mass that lacks the pseudo capsule and fat ring sign.[1]

Prednisolone with or without tamoxifen is the first-line treatment for symptomatic MP, with a positive response rate of 60% at 12-16 weeks.[5] Other treatment options with different degrees of efficacy include colchicine, azathioprine, progesterone, cyclophosphamide, and thalidomide. Innovative treatments include thalidomide and low-dose naltrexone. According to the findings of a small case study, hormonal and immunomodulatory medications are also prescribed. However, they might have substantial side effects. Except for treating focal intestinal blockage caused by fibrotic types of the disease, surgical intervention is not curative and should be avoided.[6]

MP is a self-limiting inflammatory lesion, with a good prognosis, fewer recurrences, and serious complications. There is still no consensus on the duration of treatment and follow-up for asymptomatic cases. Due to the rarity of the disease, adequate randomized clinical trials on the most effective treatment approaches and the duration of prescribing immunosuppression have not been conducted.

Authors' Contribution

AK.G: Patient management and drafting, N.S: Data collection and reviewing of the manuscript. All authors have read and approved the final manuscript and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Conflict of Interest: None declared.

Keywords ● Mesentery ● Panniculitis ● Colon descending ● Inflammation

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IJMS staff would check the structure and content of manuscripts to ensure compliance with standard structures (based on the type of study), the Journal's scope, standard guidelines, quality, novelty, and adherence to ethical issues. In this step, articles would be screened and triaged in the shortest possible time. As a result, such screening would assist the editor in making fair final decisions on articles and ultimately improve the final quality of published articles.

Peer-Review Process:

Submitted articles are primarily evaluated by our statisticians who check the articles for any methodological flaws, format, and their compliance with the Journal's instructions. Then, a submission code will be allocated and all the future contacts should be based on this code. Through a single-blind review, the articles will be reviewed by at least two external (peer) reviewers. Their comments will be passed to the authors and their responses to the comments along with the reviewers' comments will then be evaluated by the Editor-in-Chief, and a final reviewer who can be a member of the Editorial Board. The final review process will be discussed in regular editorial board sessions and on the basis of the comments, and the Journal's standards, the Editors-in-Chief will decide which articles should be published.

It should be noted that articles submitted by the staff and editors of the IJMS will also be subjected to peer review and the editor plays no role in the review or decision.

Editorial Independence:

Although the IJMS is sponsored financially by Shiraz University of Medical Sciences, it benefits from editorial freedom. The editors evaluate and accept articles based only on significance, originality, validity, and adherence to the aims and scope of the Journal.

Our editorial policy is consistent with the principles of editorial independence presented by the World Association of Medical Editors (WAME).

Ethical Considerations:

The Journal is a member of the Committee on Publication Ethics (COPE). COPE's flowcharts and guidelines are approached in confronting any ethical misbehavior. The Journal also follows the guidelines mentioned in the *Recommendations for the Conduct, Reporting, Editing and Publication of Scholarly Work in Medical Journals* issued by the International Committee of Medical Journal Editors (ICMJE).

- **Human & Animal Rights Statement:**

Research involving human subjects, human material, or human data, must have been performed in accordance with the Declaration of Helsinki and must have been approved by an independent local, regional or national review body (e.g., ethics committee, institutional review board).

Manuscripts describing any experimental research on animals should include a statement of approval by the Ethical Committee of the institute where the work

was done, mentioning that the study was carried out according to the legal requirements of the relevant local or national authority. Before papers describing animal studies are accepted for publication in the Iranian Journal of Medical Sciences, the authors must convince the editors that the work conformed to appropriate ethical standards. The care and use of experimental animals should comply with acceptable international animal welfare guidelines and policies. Further guidance on animal research ethics is available from the Guideline for the Care and Use of Laboratory Animals in Iran. For reports of such studies, authors should refer to the ARRIVE guidelines checklist.

- **Informed Consent:**

All patients and participants in a research project should be thoroughly informed about the aims of the study and any possible side effects of the drugs and interventions. Written informed consent from the participants or their legal guardians is necessary for any such studies. The Journal reserves the right to request the related documents. Articles that require informed consent should contain related statements in the "Methods" section.

- **Authorship:**

According to the *Recommendations for the Conduct, Reporting, Editing and Publication of Scholarly Work in Medical Journals*, released by the ICMJE, an "Author" is generally considered to be someone who simultaneously meets the following conditions 1, 2, 3, and 4.

1- Substantial contributions to the conception or design of the work; or the acquisition, analysis, or interpretation of data for the work; AND

2- Drafting the work or revising it critically for important intellectual content; AND

3- Final approval of the version to be published; AND

4- Agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

From September 1, 2022:

1. Manuscripts submitted to IJMS with co-first authors, will be processed only when the following conditions are met:

a) The manuscript should be original.

b) All contributing authors should qualify for authorship, and should have sufficient participation in the work. (Authorship credit should be met.)

c) The dimensions of work require several authors with different specialty, who provide the same or equal contribution(s) to the research work.

2. With regard to the corresponding Author, IJMS accepts only one corresponding author, who is responsible for all the correspondence with the journal, both before and after the publication of the manuscript.

- **Conflict of Interest:**

All authors and reviewers must inform us about any kinds of "Conflict of Interest" (such as financial, personal, political, or academic) that would potentially affect their judgment. Each author must fill the uniform disclosure form available through the below link and upload it at the time of submission:
(http://www.icmje.org/disclosure-of-interest/)

- **Plagiarism:**

Authors are not allowed to utilize verbatim text of previously published papers or manuscripts submitted elsewhere. All submitted manuscripts will be checked for possible similarity in any part of the work by iThenticate plagiarism checker. Based on the amount of plagiarism detected in a manuscript, it will be passed to the authors and ask them to rephrase copied phrases or include as direct quotations with references. COPE's flowcharts and guidelines are approached in cases in which plagiarism is detected.

- **Data Fabrication/Falsification:**

Falsification is the practice of omitting or altering research materials, data, or processes so that the results of the research are no longer accurately reflected. Fabrication is the practice of inventing data or results and reporting them in the research. Both of these misconducts are fraudulent and seriously alter the integrity of the research. Therefore, articles must be written based on original data and the use of falsified or fabricated data is strongly prohibited. In confronting cases with fabricated or falsified data COPE's flowcharts and guidelines are followed.

Researchers who perform randomized controlled or clinical trials (RCTs) are advised to keep their original data at hand. The Journal keeps the right to ask for raw data even after publication.

- **Image Manipulation:**

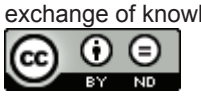
The IJMS encourages authors to send their original images. All digital images in manuscripts accepted for publication will be checked for inappropriate manipulation. No specific feature within an image may be enhanced, obscured, moved, removed, or introduced. Adjustments of brightness, contrast, or color balance are acceptable as long as they are applied to the entire image and do not misrepresent any information present in the original, including the background. The editors will request the original data from the authors to compare the manipulated figures in cases suspected of inappropriate manipulation.

- **Copyright:**

If a manuscript contains any previous published image or text, it is the responsibility of the author to obtain authorization from copyright holders. The author is required to obtain and submit the written original permission letters for all copyrighted material used in his/her manuscript.

- **Open Access Policy:**

This journal provides immediate open access to its content on the principle that making research freely available to the public supports a greater global exchange of knowledge.



- **Retraction Policy:**

The IJMS uses the COPE flowchart for retraction of a published article to determine whether a published article should be retracted.

- **Complaints and appeals**

The "Iranian Journal of Medical Sciences" responds promptly to complaints and ensures there is a way for dissatisfied complainants to take complaints further by sending an email to editor-in-chief of the journal. We follow the COPE's guidelines and flowcharts for handling complaints against the journal, its staff, editorial board or publisher.

Requirements for Different Types of Articles

Original articles: Should contain a structured abstract, 3-5 keywords, introduction, materials (patients) and methods, results, discussion, conclusion, acknowledgement (if applicable), and references sections, and maximum 4 tables and 4 figures. The length should not exceed 3000-3500 words excluding the references, abstract, figures and tables. 15-40 references should be stated for original articles.

-Clinical trials- The format is similar to original articles. However, the CONSORT flow diagram should be added as a figure. RCTs should be registered at any RCT Registry approved by the WHO and their registration number should be mentioned in the title page. RCTs done in Iran must be registered at www.irct.ir.

Brief reports: Should contain a non-structured abstract, 3-5 keywords, introduction, materials and methods, results, discussion, conclusion, acknowledgement (if applicable), and references sections, and maximum 2 tables and figures. However, the word count should not exceed 2000 words. 10-15 references should be stated.

Case reports: Should contain a non-structured abstract, 3-5 keywords, introduction, case presentation, discussion, conclusion, acknowledgment (if applicable), and references sections, and maximum 1 table and 3 figures. 5-10 references should be stated. Case reports should optimally be accompanied by relevant figures to document findings. Informed consents should be obtained from the patients to report their cases. The Journal keeps the right to ask for original signed informed consents.

Review articles:

-Narrative reviews- Should contain at least 60 references. Abstract should be non-structured. The word count should not exceed 4500 words. Narrative reviews should critically assess the current knowledge of the field.

-Systematic reviews- Systematic reviews of RCTs or Observational Studies will be accepted by the Journal. The protocol of the study should adhere to PRISMA or MOOSE guidelines for systematic reviews of RCTs or Observational studies, respectively. Up to 40 references can be stated. Abstracts should be structured.

Letters to the Editor: Letters should be less than 750 words. Letters discussing articles published in the IJMS should be submitted at most within 4 months after the publication of the main article. Letters will undergo peer review and will be edited for clarity. Up to 5 references should be stated.

Editorials: From September 1, 2022, are mostly commissioned and discuss any important issues in general medicine. Between 700-900 words and up to 10 references can be stated. Only one co-author is permitted.

Photo clinics: Figures that contain a significant medical point can also be accepted. Photo clinics should contain one or two high quality figures and a description of the figures no more than 500 words. Up to 5 references should be stated.

Paper Preparations:

Cover letter should contain the rationale of performing the research and selecting the IJMS as well as a statement that you will not resubmit your article to another journal until the reviewing process will be completed. Also please indicate whether the authors have published or submitted any related papers from the same study.

Title page of the article should include 1) the title of the article; 2) authors' names; 3) name of the institution where the work was done; 4) running title (short form of the main title presented on the top of published pages); and 5) complete mailing address, telephone/fax numbers, and email address of the corresponding author. This page is not numbered.

What's Known and What's New boxes should contain at most 50 words stating the current knowledge on the topic of your article according to previous studies (what's known), and the novel points/results your article adds to the literature (what's new).

Abstract should be structured for original articles providing the background/objective of the study, methods, results, and conclusion. It should be about 250-300 words altogether. Number this page as page 1. Abstracts of other types of contributions should be non-structured providing the essential information.

When abstracting a review article, a concise summary of the salient points should be addressed.

Preferably, abbreviations should not be mentioned in the abstract.

Keywords are used for indexing purposes; each article should provide three to five keywords selected from the Medical Subject Headings (MeSH).

Introduction should contain a short background of previous studies and possible gaps in the literature, and specify the purpose and objective of the study or observation.

Methods section must indicate clearly the steps taken to acquire the data. Be sure that it includes only information that was available at the time the plan or protocol for the study was written. It should be detailed (including: controls, inclusion and exclusion criteria, etc) and may be separated into subsections. Repeating the details of standard techniques is best avoided.

For reports of randomized controlled trials, authors should refer to the CONSORT statement (http://www.consort-statement.org/).

Reporting guidelines such as STROBE, STARD, and PRISMA would help you report high quality research

and to provide all required information and evidence for related methodology. EQUATOR Network website would help you in using these guidelines.
The software used for statistical analyses and description of the actual method should be mentioned.
Results should be presented in a chronological sequence in the text, table, and illustration. Organize the results according to their importance. They should be based on your own findings.
Tables and illustrations must be cited in order which they appear in the text, using Arabic numerals. Tables should be simple and should not duplicate information in the text of the paper. Figures should be provided only if they add further information. For radiographic films, scans, and other diagnostic images, as well as pictures of pathology specimens or photomicrographs, send high resolution figures in JPEG or Bitmap format. Type legends for illustrations on a separate page, and explain the internal scale and identify the method of staining in photomicrographs. Arrows should be put in place for specific sections of the pathology figures and radiographs.
Discussion should challenge the findings of the study with other available evidence in the format of arguments and counterarguments. It should emphasize the new and important aspects of the study and the conclusions that follow them. Possible mechanisms or explanations for these findings should be explored. The limitations of the study and the implications of the findings for future research or clinical practice should be explored.
Conclusion should state the final result and recommendation that the author(s) has (have) reached. Such results and recommendations should be derived from the researcher's own study and the results of other studies should not be stated in this section.
Supplementary materials such as movie clips, questionnaires, etc may be published on the online version of the Journal.
Any technical, general, financial, and material support or contributions that need acknowledging but do not justify authorship, can be cited at the end of the text as **Acknowledgment.**
References should be complied numerically according to the order of citation in the text in Vancouver style. The numbers of references should preferably not exceed 40 for original articles, 15 for brief, and 10 for case reports. References should optimally be prepared with EndNote software.

For the references credited to more than 6 authors please provide the name of the first six authors and represent the remaining authors by the phrase "et al."

For various references please refer to "the NLM style guide for authors, editors, and publishers".

Listed below are sample references. Moreover, IJMS has its own EndNote style. Authors are advised to prepare their references based on this style. This style is available at this link.

Journal Article:

- Gaydess A, Duysen E, Li Y, Gilman V, Kabanov A, Lockridge O, et al. Visualization of exogenous delivery of nanoformulated butyrylcholinesterase to the central nervous system. Chem Biol Interact. 2010;187:295-8. doi: 10.1016/j.cbi.2010.01.005. PubMed PMID: 20060815; PubMed Central PMCID: PMC2998607.
- Javan S, Tabesh M. Action of carbon dioxide on pulmonary vasoconstriction. J Appl Physiol. In press 2005

Complete Book:

- Guyton AC: Textbook of Medical Physiology. 8th ed. Philadelphia, PA, Saunders, 1996.

Chapter in Book:

- Young VR. The role of skeletal muscle in the regulation of protein metabolism. In Munro HN, editor: Mammalian protein metabolism. Vol 4. San Diego; Academic; 1970. p. 585-674.

Language and Style:
All submitted manuscripts must be "spell checked" and "grammar checked." Furthermore, the manuscripts should be written in proper English (American or British usage is acceptable, but not a mixture of both). If the Journal feels the submitted manuscript has major grammatical and spelling errors, the Editor may request the author(s) to improve the English of their work to minimize potential grammatical or spelling errors while still conforming to accurate scientific English standards. Therefore, to continue with the article process, the authors should use/ employ the English Language Editing service and provide a valid certificate.

Concerning manuscripts that are in the acceptance process, if the English language of the manuscript is not of publication quality and requires major improvement, the Editor may request the author(s) to use a professional translation and Editing Service.

All materials should be double-spaced and pages should be numbered. Abbreviations should be standard and used just in necessary cases, after complete explanations in the first usage. The editorial office reserves the right to edit the submitted manuscripts in order to comply with the Journal's style. In any case, the authors are responsible for the published material.

Correction of Errata:
The Journal will publish an erratum when a factual error in a published item has been documented.

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