

Proteomics Study of Mesenchymal Stem Cell-Like Cells Obtained from Tumor Microenvironment of Patients with Malignant and Benign Salivary Gland Tumors

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Received: 24/October/2020, Accepted: 13/February/2021

Abstract

Objective: Salivary gland tumors (SGTs) show some aggressive and peculiar clinicopathological behaviors that might be related to the components of the tumor microenvironment, especially mesenchymal stem cells (MSCs)-associated proteins. However, the role of MSCs-related proteins in SGTs tumorigenesis is poorly understood. This study aimed to isolate and characterize MSCs from malignant and benign tumor tissues and to identify differentially expressed proteins between these two types of MSCs.

Materials and Methods: In this experimental study, MSC-like cells derived from benign (pleomorphic adenoma, n=5) and malignant (mucoepidermoid carcinoma, n=5) tumor tissues were verified by fluorochrome antibodies and flow cytometric analysis. Differentially expressed proteins were identified using two-dimensional polyacrylamide gel electrophoresis (2DE) and Mass spectrometry.

Results: Results showed that isolated cells strongly expressed characteristic MSCs markers such as CD44, CD73, CD90, CD105, and CD166, but they did not express or weakly expressed CD14, CD34, CD45 markers. Furthermore, the expression of CD24 and CD133 was absent or near absent in both isolated cells. Results also discovered overexpression of Annexin A4 (Anxa4), elongation factor 1-delta (EF1-D), FK506 binding protein 9 (FKBP9), cytosolic platelet-activating factor acetylhydrolase type IB subunit beta (PAFAH1B), type II transglutaminase (TG2), and s-formylglutathione hydrolase (FGH) in MSCs isolated from the malignant tissues. Additionally, heat shock protein 70 (Hsp70), as well as keratin, type II cytoskeletal 7 (CK-7), were found to be overexpressed in MSCs derived from the benign ones.

Conclusion: Malignant and benign SGTs probably exhibit a distinct pattern of tissue proteins that are most likely related to the metabolic pathway. However, further studies in a large number of patients are required to determine the applicability of identified proteins as new targets for cancer therapy.

Keywords: Mass Spectrometry, Mesenchymal Stem Cells, Two-Dimensional Polyacrylamide Gel Electrophoresis

Cell Journal (Yakhteh), Vol 24, No 4, April 2022, Pages: 196-203

Citation: Haghshenas MR, Erfani N, Khansalar S, Khademi B, Ashraf MJ, Razmkhah M, Ghaderi A. Proteomics study of mesenchymal stem cell-like cells obtained from tumor microenvironment of patients with malignant and benign salivary gland tumors. Cell J. 2022; 24(4): 196-203. doi: 10.22074/cellj.2022.7844. This open-access article has been published under the terms of the Creative Commons Attribution Non-Commercial 3.0 (CC BY-NC 3.0).

Introduction

Pleomorphic adenoma (PA) is the most common type of benign parotid gland tumors, characterized by a high recurrence rate following primary surgery. Although classified as a benign tumor, it can display some peculiar behaviours as well as problems in the clinical course due to its tendency to recur and risk of malignant transformation and distant metastases (1).

Mucoepidermoid carcinoma (MEC) is the most frequently diagnosed malignancy in both adults and children. It comprises 34% of malignant salivary gland tumors (SGTs) (2). MECs are with varying potential for aggressive behaviour and are more likely to show neural invasion (3).

The tumor progression and cancer behaviour in different types of cancers, including SGTs, are affected by

the components of the tumor microenvironment (TME), particularly mesenchymal stem cells (MSCs) (4, 5). Malignant SGTs are epithelial tumor cells, but they can easily be disseminated to local or distant organs under a process named epithelial-mesenchymal transition (EMT).

MSCs were found to be recruited to salivary gland microenvironment, converted into cancer-associated fibroblasts (CAFs)-like phenotype, and then disband cell-cell connection in SGT cells. The consequences of such conversions and interactions are cancer dissemination (6). There is strong evidence that MSCs play an important role in cancer stem cell survival and can regulate their self-renewal (7, 8). MSC-activated immune responses induce regulatory T cells and regulatory B cells while suppressing proliferation, maturation, and differentiation of T and B lymphocytes (7, 9). In addition to MSCs-based immunomodulation, they

had been described as regulatory players in the metabolic reprogramming of cancer cells (10). It is suggested that MSCs via intracellular/surface proteins or soluble factors contribute to immune suppression and cancer progression.

The proteomic expression of tumor-MSCs is less investigated in comparison to those expressed by tumor cells. Proteomics emerged as a large-scale screening tool for protein discovery. The main methods for proteomics studies are two-dimensional gel electrophoresis (2-DE) and mass spectrometry (MS) (11). MSCs proteomic analysis may provide valuable data that are particularly controlled by MSCs such as proteins involved in immune suppression, cancer metabolism, and cancer development (12, 13).

Therefore, to develop molecular aspects of SGTs carcinogenesis this study aimed to isolate and characterize MSCs from malignant and benign tumor tissues and to identify differentially expressed proteins between these two types of MSCs.

Materials and Methods

Sample collection and MSCs isolation

The study was approved by the local Ethics Committee of Shiraz University of Medical Sciences, Shiraz, Iran (IR.SUMS.REC.1399.675). Written informed consent was obtained from each member before sampling. In this experimental study, MSCs were individually isolated and cultured from tumor tissues derived from patients with PA (n=5) and MEC (n=5) subtypes in a manner named "explants" culture. Briefly, the specimens were washed and minced in very small pieces with a scalpel and distributed into 6-well tissue culture plates and incubated in a 37°C-5% CO₂ incubator in the presence of Dulbecco's modified Eagle's medium (DMEM), 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. When the cells reached 60-70% confluency, they were harvested and transferred into a larger culture flask. Crystal violet staining (0.5% crystal violet in methanol) of MSCs isolated from malignant and benign tumor tissues are shown in Figure 1.

Flow cytometry analysis

To verify and compare isolated cells obtained from tumor tissues patients with PA and MEC subtypes, the attached cells (in passages 3 to 4) were harvested by treatment with 1% trypsin-EDTA. After washing, the cells were incubated with CD105, CD24, CD45, CD34 and CD14 antibodies (FITC mouse anti-human), CD44, CD133, and CD166 antibodies (PE mouse anti-human), and CD90 and CD73 antibodies (APC mouse anti-human) at 4°C for 30 minutes in the dark. The data were collected on BD FACSCalibur flow cytometer and then analyzed by BD CellQuest Pro software package. The respective isotype control antibodies were used in separated tubes as negative controls. The antibodies, the instrument, and the software package were all from BD Biosciences, USA.

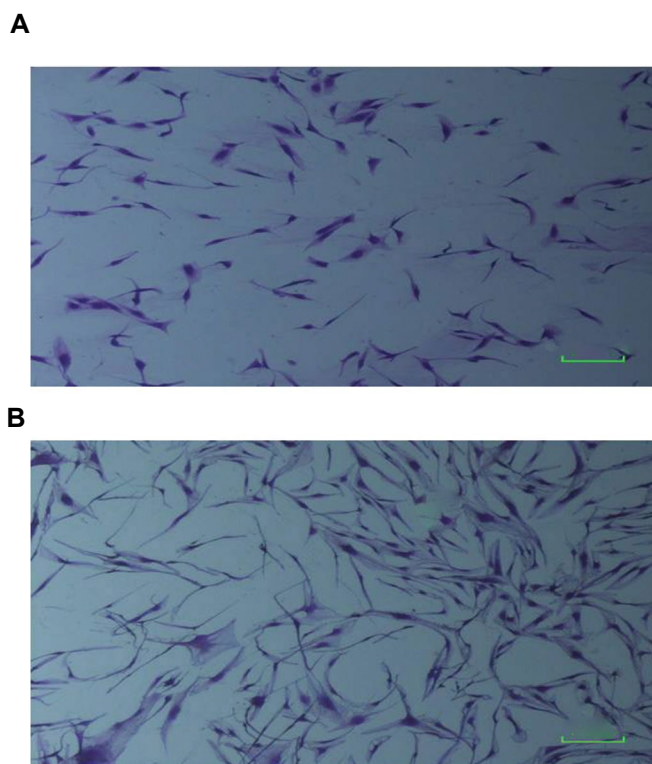


Fig.1: Mesenchymal stem cell (MSC)-like cells isolated from tumor microenvironment of patients with salivary gland tumors. Crystal violet staining of MSC-like cells isolated from **A.** Malignant and **B.** Benign tumor tissues in passage 4. The metric units are 100.00 μ m.

Protein extraction, isoelectric focusing, SDS-page electrophoresis, and gel staining

Cell pellets were lysed by a lysis buffer as previously described (12, 13). The protein concentration was calculated by the Bradford assay (14). The first dimension of electrophoresis (or IEF) was done using GE18-cm IPG gel strips (pH=3-10 NL) to separate proteins based on their isoelectric point (pI). 500 μ g of each sample was added to rehydration buffer (7 M urea, 2 M thiourea, 2% CHAPS, and 0.002% bromophenol blue), 0.001g of dithiothreitol (DTT), and 0.5% (1.7 μ l) GE immobilized pH gradient (3-10, NL) buffer. The mixture was applied on IPG strips and incubated for 45 minutes at 20°C. Strips were covered by cover fluid and then actively rehydrated for 16 hours at 50 volt (20°C). After rehydration, the cover fluid was changed, and strips were focused for 70000 volt-hour at 20°C. In the second dimension of electrophoresis, proteins were separated based on their molecular weight (MW) by the protean II xi cell system. Before the second dimension, focused strips were equilibrated, reduced, and alkylated in the presence of an equilibration buffer (12, 13). To minimize the variation in analysis, the same parameters were used for each gel, and a control sample was repeatedly run and stained. IPG gel strips were fixed in a fixative solution and coomassie brilliant blue (CBB) G-250 dye solution was used to visualize protein spots on gels.

Gels imaging, spots detection, protein identification, and database analysis

The stained gels were scanned at 300 dpi resolution, and then protein spots were analyzed using the Prodigy SameSpots software package (Nonlinear Dynamics). Differential spots were cut from the gel, and then sent to York University (York, UK) to be identified by Matrix-Assisted Laser Desorption Ionization Tandem Time-of-Flight mass spectrometry (MALDI-TOF-TOF MS). In the present study, MASCOT protein scores ≥ 62 were considered statistically significant ($P < 0.05$). On the other hand, individual ions scores ≥ 62 indicate identity or extensive homology. PANTHER classification database (<http://pantherdb.org>), as well as UniProtKB database (<https://www.uniprot.org/>), were used to analyze the molecular function and biological process of identified proteins.

Statistical analysis

Prodigy SameSpots software package (Version 1.0, Nonlinear Dynamics, UK) automatically calculated normalized volume (% expression), and further statistical analysis was performed by Mann–Whitney U-test and t test (SPSS Inc, Version 11, Chicago IL, USA). $P < 0.05$ was considered significant level in all cases.

Results

The clinical and pathological characterization of the patients

Five malignant tumor tissues with MEC tumor types and 5 benign tumor tissues with PA tumor types obtained from patients with SGTs were enrolled in our study. Clinicopathological characterization of each patient with malignant and benign SGTs is shown in Table 1.

Flow cytometric analysis of MSC-like cells

According to the forward (F-) and side (S-) scatter (SC) dot plots, both MSC-like cells isolated from malignant and benign tumor tissues have a similarity in granularity and size (Fig.S1, See Supplementary Online Information at www.celljournal.org). MSCs isolated from malignant and benign tumor tissues strongly expressed the characteristic MSCs markers such as CD44, CD73, CD90, CD105, and CD166, whereas did not express or rarely expressed CD14, CD34, and CD45. The expression of CD24 and CD133 was absent or nearly absent in both isolated cells. Flow cytometric analysis of MSC-like cells is shown in Table 2.

Table 1: Clinicopathological characterization of patients with malignant (MEC) and benign (PA) salivary gland tumors

Cases	Tumor type	Grade/Stage	Tumor location	Age/Gender
M1	MEC	Poorly/III	Parotid	55/Male
M2	MEC	Moderately/I	Parotid	27/Female
M3	MEC	Well/II	Parotid	52/Male
M4	MEC	Poorly/IV	Parotid	57/Male
M5	MEC	Well/IV	Parotid	73/Female
P1	PA	-	Parotid	46/Female
P2	PA	-	Parotid	52/Male
P3	PA	-	Parotid	45/Male
P4	PA	-	Parotid	53/Female
P5	PA	-	Parotid	39/Female

Table 2: Mesenchymal specific markers in MSC-like cells isolated from malignant (MEC) and benign (PA) tumor tissues

MSCs types	CD14	CD34	CD45	CD90	CD73	CD105	CD166	CD44	CD24	CD133
Mucoepidermoid carcinoma (MEC)	1.6 ± 0.27	3.3 ± 0.61	1.4 ± 0.27	92 ± 1.9	94.7 ± 2.7	93.2 ± 4.7	91 ± 2.3	94.2 ± 1.1	1.9 ± 0.33	2.1 ± 0.37
Pleomorphic adenoma (PA)	1.6 ± 0.40	3.5 ± 1.2	2.4 ± 0.57	91.7 ± 0.88	93.5 ± 2.3	97.9 ± 0.48	93 ± 2.4	95.1 ± 0.97	2.5 ± 0.58	0.84 ± 0.18

Mean ± standard deviation (SD) were calculated using the SPSS program (SPSS Inc, Chicago IL, USA).

Differentially expressed proteins between MSC-like cells isolated from malignant (MEC types) and benign (PA types) tumor tissues

After validation of MSC-like cells with mesenchymal markers, 2-DE and MS analysis were performed. Our results indicated that despite the overall similarity in protein expression pattern, at least 11 different protein spots were differentially, and reproducibly expressed. The location of differential spots on gels were matched to the available proteome map before MS. Of them, 8 spots were identified by a significant

score (score ≥ 62 and $P < 0.05$). The identified proteins were summarized in Table 3. Two spots were identified with scores < 62 and one spot did not match the database significantly. Our results indicated that the expression of PAFAH1B, FGH, TG2, FKBP9, Anxa4, and EF1-D significantly elevated among MSC-like cells derived from malignant cases. In comparison, the expression of Hsp70 and CK-7 was significantly higher in MSC-like cells derived from benign cases. The spot details, molecular function, and biological process of identified proteins are presented in Figures 2 and 3, and Table 3.

Table 3: Details of differential proteins in MSC-like cells derived from malignant and benign tumor tissues

Spot No.	Protein name/Gene name	Accession No.	Calculated pI/ Nominal mass (Mr-KDa)	Score	Matches peptides number/ Sequences number	Coverage %	Molecular function and biological process based on PANTHER classification database and UniProtKB
1	Cytosolic platelet-activating factor acetyl hydrolase type IB subunit beta (PAFAH1B2)/ <i>PAFAH1B2</i>	P68402	5.57/25.72	127	2/2	12	Protein modifying enzyme, Ether lipid metabolism, Metabolic pathways
2	S-formylglutathione hydrolase (FGH)/ <i>ESD</i>	P10768	6.54/31.95	114	1/1	7	Metabolic pathways, Catalytic activity
3	Type II trans glutaminase (TG2)/ <i>TGM2</i>	P21980	5.11/78.42	155	3/2	4	Catalytic activity, Cellular process, Metabolic process
4	FK506 binding protein 9 (FKBP9)/ <i>FKBP9</i>	O95302	4.91/63.50	348	6/6	10	Calcium ion binding, Protein folding
5	Annexin A4 (Anxa4)/ <i>ANXA4</i>	P09525	5.84/36.08	255	4/4	15	NF-kappaB signaling, Epithelial cell differentiation, phospholipase inhibitor activity, Negative regulation of the apoptotic process
6	Keratin, type II cytoskeletal 7 (CK-7)/ <i>KRT7</i>	P08729	5.40/51.41	304	5/5	13	Cornification, DNA synthesis
7	Heat shock protein 70 (Hsp70)/ <i>HSPA9</i>	P38646	5.87/73.92	442	6/6	11	ATPase activity, Cellular response to unfolded protein
8	Elongation factor 1-delta (EF1-D)/ <i>EEF1D</i>	P29692	4.90/31.21	62	1/1	8	Binding, Molecular function regulator, Cellular process, Metabolic process, I-kappaB kinase/NF-kappaB signaling

UniProt accession number. pI; Isoelectric point.

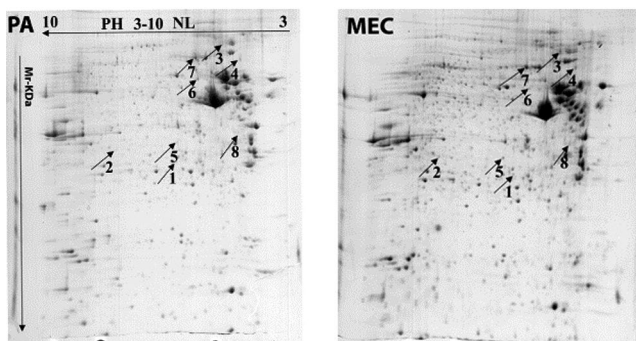


Fig. 2: Differential spots on coomassie blue staining gels. Spot numbers are the same as those in Table 3. Left side: Pleomorphic adenoma (PA), Right side: Mucoepidermoid carcinoma (MEC).

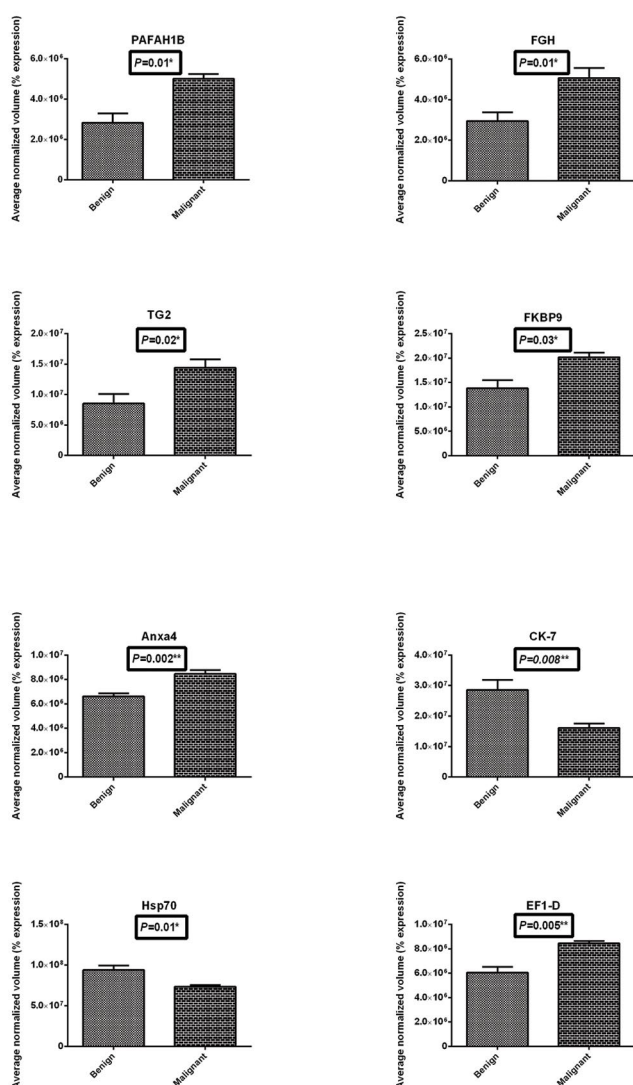


Fig. 3: Comparison of average normalized volumes of identified proteins between MSC-like cells isolated from malignant and benign tumor tissues. The expression pattern of PAFAH1B, FGH, TG2, FKBP9, EF1-D, and Anxa4 was found to be significantly elevated in MSC-like cells derived from malignant tissues (MEC tumor type), n=5. The expression of CK-7 and Hsp70 was significantly higher in MSC-like cells derived from benign ones (PA tumor type), n=5. Number represents average normalized volume. MEC; Mucoepidermoid carcinoma, PA; Pleomorphic adenoma, *; Difference, and **, Difference are significant at <0.05 and <0.01 levels respectively.

Discussion

In this study, we isolated MSC-like cells from malignant and benign tumor tissues (MEC and PA tumor types), verified them using mesenchymal specific markers, and then investigated the differentially expressed proteins by 2-DE in combination with mass spectrometry. Microscopic images, as well as surface staining of cells derived from both malignant and benign tumor tissues by a panel of fluorochrome antibodies, indicated that morphological features and mesenchymal specific markers of both isolated cells were quite similar to each other and with other known MSCs (12, 13). Since both MSC-like cells were obtained from the same source, parotid tissue, they expressed similar mesenchymal markers (15).

In this regard, both highly expressed CD44, CD73, CD90, CD105, and CD166 markers, and did not express or rarely expressed markers such as CD14, CD34, and CD45. Based on the International Federation of Adipose Therapeutics and Sciences (IFATS) and International Society for Cellular Therapy (ISCT), MSCs characterization are mostly restricted to positive expression of a panel of classical cell surface markers including CD44, CD73 (L-VAP-2), CD90 (Thy-1), and CD105 (Endoglin), and lack of CD14, CD34 and CD45 (leukocyte common antigen) markers (16). CD24 is a receptor that interacts with P-selectin to promote tumor development and metastatic activity (17). CD166 (ALCAM) and CD133 (Prominin-1) could be served as potential cancer stem cell markers (18, 19).

In our study, both benign and malignant MSC-like cells seldom expressed CD24 and CD133, while they were positive for CD166. Furthermore, to determine the molecular pathology behind the malignant and benign tumors, we compared the protein expression pattern between MSC-like cells isolated from malignant tissues (MEC tumor type), and MSC-like cells isolated from benign ones (PA tumor type), and we finally identified differentially expressed proteins. Our results indicated that although MSC-like cells from both tumor tissues expressed similar markers, they exhibited differential expression of proteins. One reason is that MSCs are probably educated by tumor cells and their mediators once they are recruited to the TME (20).

It has been shown that cancer behavior is strongly regulated by the components of the TME through networks of the protein-protein interactions (21). A large number of physiological and pathological processes are strictly controlled by these protein-protein interaction networks, and any changes in the protein expression have a direct influence on cancer behavior and tumor progression (22). Since biological and clinical behaviors of benign and malignant tumors are different, therefore, the protein expression pattern is expected to be dissimilar in these types of cells. In head and neck squamous cell carcinoma, it has been shown that expressed proteins are implicated in various cellular processes including tumor growth, apoptosis, cell death, cell cycle, tumor proliferation,

invasion, migration, metastasis, and response to therapy (23).

Detail characterizations of differentially expressed proteins in cancer patients not only are shedding light on cancer biology, but also are implicated in predicting SGTs aggressiveness, distinguishing malignant SGTs from benign ones, and/or developing new targets for cancer therapy (24, 25). Since benign PA can be misdiagnosed cytologically with ACC or MEC, differentially expressed proteins may serve as complementary markers to discriminate SGTs benign from malignant ones (25).

Database analysis, in our study, indicated that identified proteins were involved in different pathways. Among them, proteins related to metabolic pathways were enriched in proteomic profiles of tumor-MSCs isolated from malignant tumor tissues. Reprogramming of cellular metabolism exhibits an important role in carcinogenesis and metastasis. Cancer cell metabolism may facilitate the deregulated uptake of glucose and essential amino acids as well as the metabolic interactions with TME to sustain cell viability and generate new biomass, situations that are associated with tumor progression (26).

It was recently reported that MSCs, as key regulators of the tumor microenvironment, play an essential role in the metabolic reprogramming of tumor cells in osteosarcoma (10). Consequently, metabolic pathways could be employed as an attractive target for therapeutic policies (27). In our study, the differential expression of proteins was in two forms; proteins overexpressed in MSC-like cells isolated from malignant tissues, and proteins overexpressed in MSC-like cells isolated from benign ones. In this regard, six proteins including PFAH1B, FGH, TG2, FKBP9, Anxa4, and EF1-D were found to be overexpressed amongst MSC-like cells isolated from malignant tumors. As mentioned, based on the PANTHER classification database and UniProtKB database analysis, the majority of identified proteins in MSC-like cells derived from malignant tissues were found to be involved in metabolic pathways. In this regard, Annexin may be involved in cancer metabolism, at least in part, through phospholipase inhibitor activity.

In breast cancer, PFAH1B3 has been shown to act as a critical metabolic driver of cancer progression. Overexpression of this enzyme in primary human breast cancer is shown to be related to poor prognosis (28). Thereby, targeting metabolic drivers can be a promising therapeutic strategy in cancer eradication (29). Another enzyme that was significantly over-expressed in MSC-like cells isolated from malignant tissues was FGH or esterase D (ESD). FGH is mainly implicated in the detoxification of formaldehyde, but little is known regarding its biological function and physiological role. It was reported that this protein might be considered as a predictive marker to determine aggressive lung adenocarcinomas in humans (30).

The other identified protein, TG2, is known as a cancer stem cell survival factor. TG2 also can regulate glucose

metabolic reprogramming in a pathway dependent on the nuclear factor (NF)- κ B (31). TG2 also is an essential factor in the osteogenesis and chondrogenic differentiation of MSCs (32, 33). Peptidyl-prolylisomerase (PPIase) families comprise of FK506 binding proteins, cyclophilin, and PIN1. The relation between PPIase families and tumor progression is not clearly defined. However, some evidence showed that FK506 binding proteins and cyclophilin modulate the transformed phenotypes in tumor cells (34). The other PPIase family member, PIN1, plays an essential role in cancer metabolism, cell mobility, and cell proliferation and promotes cancer stem cells (34, 35). EF1D exerts a role in the elongation step of translation and metabolic process and is frequently overexpressed in human tumor cells (36). However, its exact role in the onset and the progression of cancer is not well understood.

In addition to mentioned proteins, AnnexinA4 (Anxa4) was found to be over-expressed in MSC-like cells isolated from malignant tumors. Anxa4 was overexpressed in many types of epithelial cancers including breast, lung, colorectal, gallbladder, gastric, ovarian, renal, prostate, laryngeal, and pancreatic cancers. Anxa4 expression may facilitate the differential diagnosis of major SGTs from thyroid cancer. Anxa4 overexpression may be associated with tumor invasion and cancer development, and may be a potential target for cancer treatment (37).

In contrast to overexpression of the mentioned proteins in MSC-like cells isolated from malignant tumor tissues, CK-7 and Hsp70 were observed to be overexpressed in MSC-like cells isolated from benign tumor tissues. CK-7 can block interferon-dependent interphase and stimulates DNA synthesis in cells. IHC analysis showed that CK-7+/CK-20-pattern is typical in both malignant and benign SGTs (38). The other over-expressed protein, HSPs, is enhanced in response to biological stress. HSPs contribute to cancer development and metastasis and may serve as biomarkers for cancer diagnosis and therapy (39). It was suggested that overexpression of Hsp70 in MSCs was associated with MSCs survival (40).

The exact roles of identified proteins in SGTs and MSCs isolated from SGTs have not been determined to date. The findings may support that MSCs can trigger metabolic dysregulation and tumor growth in malignant SGTs as reported in osteosarcoma (10). However, the current study is an explorative phase of proteomics studies that need to be verified and validated by antibody-based methods such as western blot in larger cohorts of individuals to determine the exact roles of MSCs-derived proteins in cancer progression and cancer therapy.

Conclusion

Our findings show that the cells isolated from malignant and benign tumor tissues similarly express the characteristic MSCs markers. However, malignant and benign SGTs probably exhibit a distinct pattern of tissue proteins that are most likely related to the

metabolic pathway. The identified proteins may exert an important role in the unfavorable behavior of SGTs and more especially in those with malignant type. Our results suggest that MSCs or their components may consider as desirable therapeutic targets in SGTs.

Acknowledgments

This study was supported by a grant from Shiraz University of Medical Sciences [Grant number 99-01-16-22976 (20593)] as well as from Shiraz Institute for Cancer Research (ICR-100-503). The authors wish to thank Dr. Seyed Mohammad Jafari at the Research Consultation Center (RCC) of Shiraz University of Medical Sciences for editing this manuscript. There was no conflict of interest among the authors.

Authors' Contributions

A.Gh., M.R., N.E.; Contributed to the conception and study design. M.R.H.; Contributed to lab experiments, flow cytometric and proteomics analysis, statistical analysis, and data interpretation. S.Kh.; Contributed to proteomics analysis. B.Kh., M.J.A.; Contributed to patient's diagnosis and sample collection. A.Gh., M.R.; Supervised the project. M.R.H.; Drafted the manuscript, and then it was revised by M.R., N.E., S.Kh., B.Kh., M.J.A., A.Gh. All authors read and approved the final manuscript.

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