Biological activity of biomarkers associated to osteosarcoma: The role of voltage-gated ion channels subunits

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Abbreviations:

BC: Betweenness Centrality
BSA: Bovine Serum Albumin
CeCa: Cervical Cancer
CPM: Counts per million
DEGs: Differentially Expressed Genes
ECM: Extracellular Matrix
EIPA: 5-(N-etil-N-isopropyl)amiloride
EMT: Epithelial-Mesenchymal Transition
FBS: Fetal Bovine Serum
FC: Fold Change
GO: Gene Ontology
HC: High Connectivity
I_K: Potassium currents
I_{Na}: Sodium currents
K_V: Voltage-Gated Potassium Channel
MMP: Matrix Metalloproteinase
Na_Vs: Voltage-Gated Sodium Channels
NCX: Na+/Ca2+ exchangers
NHE1: Na+/H+ exchanger-1
PCa: Prostate Cancer
PDBu: phorbol 12, 13-dibutyrate
PPI: Protein-Protein Interaction
RIN: RNA Integrity Number
RNA-seq: RNA sequencing
RT-qPCR: Quantitative RT-PCR
TEA: Tetraethylammonium
TTX: Tetrodotoxin
TTX-R: TTX resistent
TTX-S: TTX sensitives
VEGFA: Vascular endothelial growth factor A

Article category: ñk

Introduction

Osteosarcoma is the most frequent primary bone tumor with an unclear etiology. Therefore, most patients are diagnosed in advanced stages. The worldwide incidence is 8-11 cases/1,000,000 in adolescents and children, due this is considered rare cancer. However it is the third most common cancer in adolescents. Additionally, osteosarcoma tumors are aggressive and highly metastatic. Consequently, 90% of patients develop pulmonary metastasis and their 5-year survival rate is 19-30%, result of the poor efficacy of treatment strategies [1,2]. For these reasons, the osteosarcoma biology understanding through genomic knowledge could result in new information of potential targets for early diagnosis of the disease. For personalized medicine, these findings represents an imperative healthy alternative. In this sense, several efforts have been made to identify signaling pathways, development processes, and expression pattern in osteosarcoma, however, it is not clear yet how the metastatic process happens in osteosarcoma [3,4].

Metastasis is the main cause of cancer-related deaths. It is defined as the dissemination and subsequent colonization of primary tumor cancer cells to distant anatomical sites. This complex and multi-step process is the result of spatial and temporal pressures on cancer cells. The first of these steps is the cancer cell invasion/migration near the primary tumor. Cancer cell migration and invasion are crucial steps that facilitate cell dissociation by the activation of cellular mechanisms that permit cell movement, adhesion, degradation of the extracellular matrix (ECM), and the losing of cell-cell adhesions [4,5].

Voltage-Gated Sodium Channels (Na_Vs) are membrane protein complexes containing a large pore-forming α-subunit that associates with smaller β-subunit(s) as a functional component. The canonical function of Na_Vs is the initiation and propagation of action potentials. However, now it is known that these channels are proteins that have a crucial role in the regulation of many physiological processes like phagocytosis, cell differentiation, migration, invasion, and motility [6].

Recently, Na_Vs subunits have been identified over-expressed in epithelial cancer cells, and its functional expression has been related to migration and invasion characteristics. For instance, in MDA-MB-23 a highly aggressive breast cancer cell line, the functional expression of $Na_V1.5$ neonatal variant enhances migration and invasiveness. The $Na_V1.5$ silencing, as well as,

blocking by tetrodotoxin (TTX) reduce both migration and invasion [7,8]. Interestingly, Brisson in 2013 reported that $Na_V1.5$ activity enhances cell invasiveness by increasing the activity of Na+/H+ exchanger-1 (NHE1). The modulation of NHE1 generates the perimembrane acidification which activates extracellular acidic cysteine cathepsins and the subsequent ECM degradation. Furthermore, these cells showed an invasive morphology promoted by alterations in F-actin polymerization through the increase of SrC kinase activity [9,10].

Furthermore, some works reported the increase mRNA expression of $Na_V1.6$, and $Na_V1.7$ in cervical cancer (CeCa) biopsies and primary cultures positives to Papilloma Virus type 16. The functional characterization by whole-cell patch-clamp using Cn2 toxin ($Na_V1.6$-specific toxin) reduce 30% of the total sodium currents, also invasiveness was reduced ~20% in presence of TTX or Cn2 suggesting the participation of $Na_V1.6$ in invasion [11]. Furthermore, heterologous expression of $Na_V1.6$ canonical isoform in C33A CeCa cell line showed a 5-fold increment in invasiveness and TTX reverses the effect. Also, it was observed an increase of matrix metalloproteinase-2 (MMP-2) activity, suggesting that the invasiveness in CeCa is modulated by $Na_V1.6$ trough the activation of MMP-2 [12].

Moreover, other channel that has been described in cancer is $Na_V1.7$. For example, its over-expression has been reported in highly metastatic prostate cancer (PCa) and CeCa cell lines, however, the cellular mechanism involved is still unknown in these cancer types [11,13]. Additionally, Xia in 2016 reported the expression of $Na_V1.7$ in tissue samples and cell lines of gastric cancer (GC), interestingly the channel expression correlated with a poor prognosis, NHE1, and oncoprotein metastasis-associated in colon cancer-1 (MACC1) expression. $Na_V1.7$ suppression results in reduction of proliferation and invasion rates of GC cell lines by the reduction of Na_V currents, the diminution of NHE1 expression, the increase of extracellular pH, and decrease of intracellular pH. In this work was suggested that MACC1 might be involved in $Na_V1.7$-dependent expression of NHE-1 [14]. Also, in human non-small cell lung carcinoma (NSCLC), the invasion was promoted by the functional expression of $Na_V1.7$ in H460 NSCLC cells. Interestingly, the activity and expression inhibition of $Na_V1.7$, by TTX and small interfering RNA respectively, reduces H460 cell invasion up to 50% [15].

Also, cancer comprises complex biological systems that require accurate and comprehensive analysis to elucidate at molecular level its development, progression, and therapy. In this

sense, RNA sequencing (RNA-seq) offers invaluable insights for cancer research and treatment, including drug resistance, heterogeneity, and biomarker discovery [16]. For example: Chen et al., in 2020 from RNA-seq identified Toll-Like Receptor 7 (TLR7) as Differentially Expressed Gen (DEG) in osteosarcoma samples, they found that its expression was significantly associated with the prognosis. Also, the gene silencing of TLR7 decreased the number of migratory and invasive osteosarcoma cell lines. they proposed TLR7 as potential target for osteosarcoma metastasis treatment [17].

In this study, we used RNA-seq to identify the pattern expression of osteoblast (hFOB1.19) and osteosarcoma (SAOS2 and SJSA1) cell lines. By comparing the gene expression we could identify DEGs involved in biological functions related to invasion and metastasis in osteosarcoma.

Methods

Culture of Cell Lines

The cell lines SAOS2 (ATCC® HTB-85™; RRID: CVCL_0548), and SJSA1 (ATCC® CRL-2098™; RRID: CVCL_1697) were used as a model for osteosarcoma, while as non-cancerous osteoblast control the hFOB1.19 (ATCC® 11372™; RRID: CVCL_3708) cell line was used. Additionally, the dermal endothelium human cell line HMEC-1 (ATCC® CRL-3243™; RRID: CVCL_0307) was used as non cancer-bone model. All cell lines were maintained according to the manufacturer's instructions with 1% of penicillin-streptomycin (Biowest L0010) at 37ºC in a CO2 incubator.

Extraction of total RNA

Total RNA was isolated from hFOB1.19, SAOS2, and SJSA1. Briefly, 3 culture dishes of each cell line were used to collect the cells, and the RNA was isolated using the Quick-RNA™ MiniPrep Kit (Zymo Research R1054) according with the manufacturer's instructions and checked by agarose gel electrophoresis.

RNA library construction and sequencing

Short-read CDNA sequencing library strategy was carry out to analyses RNAm expression on platform Illumina Genome Analyzer GAIIx. The extraction of RNA, mRNA enrichment, RNA fragmentation, cDNA synthesis, fragmentation and amplification and sequencing were used as manufactures recommendations. Briefly, to sequence the polyadenylated fraction of RNA, total RNA was isolated from osteosarcoma and osteoblast cell lines. The quality of total RNA was analyzed by RNA Integrity Number (RIN), samples with high RIN (>8) were used for library construction, 2µg of total RNA was utilized for each sequencing library, then the libraries were sequenced. Two biological replicates for each cell line were sequenced, and the single-end RNA-seq was employed.

Read mapping, transcript assembly, and expression level estimation

The reads obtained by sequencing were cleaned with Trim Galore! Software (Babraham Bioinformatics-Babraham Institute), this consisted of the elimination of adapters and sequences with PHRED value <20. The reads conserved were mapped to the human reference genome (*Homo sapiens* GRCh38.p12 RefSeq: GCF_000001405.33 from NCBI) and quantified with Kallisto-Pachter Lab [18]. The quantification values were reported in counts per million (CPM). The CPM value of 2 was used as a threshold for minimum gene expression. Therefore, genes expressed at CPM values >2 were considered to expression analysis. DEGs between osteosarcoma and osteoblast cell lines were identified using R software and limma package [19,20]. The DEGs were filtered according to the threshold of p-value <0.05 and, fold change (logFC) value >2 (considering the housekeeping genes values ~1.5 logFC) [21,22]. The volcano maps of DEGs were plotted using ggplot R package [23].

Gene Functional enrichment analysis

To explore the biological function of relation networks for cellular processes of identified DEGs, a functional enrichment analysis of Kyoto Encyclopedia of Genes and Genomes (KEGG) and Gene Ontology (GO) in the category of cellular component (CC) were conducted with pathfindR R package [24]. A logFC value of 2.0 and p-value <0.05 were considered for active-subnetwork-oriented enrichment analysis. The pathways related with DEGs were filtered considering the cancerSEA pathways associated with invasion, migration, and epithelial-mesenchymal transition (EMT) [25].

PPI network construction

The DEGs linked to cancer pathways reported in CanserSEA were used to construct a protein-protein interaction (PPI) network. The Search Tool for the Retrieval of Interacting Genes/Proteins (STRING 11.5) database was used to predict the interactions among protein products of DEGs, considering a high confidence score ≥0.7 [26]. Gephi and igraph R package [27,28] were used to reconstruct, analyze, and visualize the network. The topological parameters used to evaluate the PPI networks were betweenness centrality (BC), degree, and hub nodes.

BC was taken as a parameter to visualize the network. Briefly, it captures how much a gene due node is in-between others. This metric is measured with the number of shortest paths that passes through the target gene node. This score is moderated by the total number of shortest paths existing between any couple of gen nodes of the graph. The target gene would have a high betweenness centrality if it appears in many shortest paths. Degree or connectivity refers to the number of edges incident on a particular node. Also, nodes with very large numbers of connections are considered hubs. For this work, a node greater than or equal to the sum of the mean and once of standard deviation of the degree distribution was considered a hub.

Quantitative RT-PCR

Previously studies have reported the presence of NaVs subunits in different types of cancer, and have also associated their presence and function with invasion and metastasis. To explore if the NaVs subunits could be related to these cancerous processes in osteosarcoma cell lines, their mRNA expression was verified through a quantitative RT-PCR (RT-qPCR). Total RNA from the hFOB1.19, SAOS2, and SJSA1 were purified with TRI Reagent (Zymo Research) and cleaned with Direct-zol RNA miniprep kit (Zymo Research). The cDNA library was generated from 2µg of total RNA in a 20µl of High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). For the PCR reaction was used TaqMan Gene Expression Master Mix (Invitrogen, cat: 4369016), 500nM of each 5' and 3' primers (Table 1), and 250 nM of the probe (Table 1), the RT-qPCR was performed in the Roche Light Cycler Nano with one step of 2 minutes at 50ºC and 10 minutes at 95ºC for enzyme activation, and then 40 cycles with denaturation at 95ºC for 15 seconds, probe annealing at 69ºC for 15 seconds, and 1 minute for primers annealing and extension at 60ºC. For negative controls were used the reaction mix without cDNA, primers or probe. All RT-qPCR reactions were performed in triplicate and cycle threshold (Ct) values were averaged. Relative expression FC was calculated using $2^{-\Delta\Delta Ct}$ [29,30]. Housekeeping Proteasome subunit beta type-2 (PSMB2) gene was used as an expression control. Amplification primers and probes for RT-qPCR were designed by Primer designing tool from NCBI [31], and by OligoCalc [32]. The primers Tm were verified by gradient PCR (data not shown), the sequences of primers and probes are shown in table1.

Migration and Invasion Assays

Transwell permeable support chamber were performed to determined the migration and invasion ability of osteosarcoma cell lines [11,12]. Briefly, $6x10^4$ cells of each cell line were seeded in the inserts using culture media with 1% Fetal Bovine Serum (FBS) in absence or presence of channel blockers: TTX 1μM (Sigma-Aldrich), Tetraethylammonium (TEA) 10mM (Sigma-Aldrich); protease inhibitors E-64 25μM (Millipore), GM6001 100μM (Millipore), and the NHE specific inhibitor: 5-(N-etil-N-isopropyl)amiloride (EIPA) 10μM (Sigma-Aldrich). The inserts were immersed in the lower chamber which contained 800μL of enriched culture medium with 10% FBS. For the cell transmembrane invasion assay, all the steps were carried out similarly to those in the migration assay except for the Matrigel coating. hFOB1.19 was used as negative control while positive migration/invasion control was used hMEC-1. After incubation at 37ºC for 24 h, the filters were removed. The cells adhering to the lower surface were fixed and stained with DAPI. To image the cells, 3 randomly selected fields in each well were photographed at the magnification of 10X and counted in three independent experiments.

Gelatin Zymography

For evaluating the activity on the MMPs, a gelatin zymography approach was performed. This method is based on analyzing the digestion of a substrate such as gelatin added to a polyacrylamide gel. This assay have several benefits: low cost, simplicity, sensitivity, densitometry semicuantitative analysis for detection of active MMPs. Firstly, $5x10^6$ cells were seeded in 24 well plates and incubated for 24 h in complete media (10% SFB), the media was removed and washed with PBS twice. Media with 1% SFB was added to the cell culture and used as problem sample, while media with 1% SFB and 100 ng/ml of phorbol 12, 13-dibutyrate (PDBu) was used as a positive control, for negative control was used media with 1% FBS and without cells. The cell culture was incubated for 40 h; after incubation 500 ul of the supernatant of each cell line and condition was added to Amicon ultra centrifuge filters (Merk Millipore) and centrifugated fo 20 min at 15,000g. The total protein of supernatant concentrate was quantified by Bradford Protein Assay, as standard bovine serum albumin

(BSA) was used. 0.1 µg of total protein samples were mixed with sample buffer (2.5% SDS, 4-µg/ml phenol blue, 40% Glycerol) under non-reducing conditions. Samples were separated by electrophoresis on 10% polyacrylamide gels containing 1ug/ml gelatin. After electrophoresis, the gels were washed three times for 25 min with 2.5% triton X-100 and incubated in activity buffer (50 mM Tris-HCl, pH 7.4; and 5 mM CaCl2) at 37 °C for 40 h. Gels were incubated in stained solution (0.25% Coomassie Brilliant Blue G-250, 10% acetic acid, 30% methanol) and proteolytic activity was visualized by destaining in methanol-acetic acid.

Electrophisiology analysis

Results

Transcriptome dynamics in osteosarcoma cell lines

For this work, the osteoblast (hFOB1.19), and OS (SAOS2 and SJSA1) cell lines were used as a model for the study of osteosarcoma. The total RNA was obtained from each cell line which was used to construct the cDNA libraries and then sequenced as previously described with Illumina Genome Analyzer GAIIx in a single-end mode. Two biological replicates for each cell line were sequenced. The reads number obtained by sequencing were $4.8x10^7$, $4x10^7$, and $5x10^7$ for hFOB1.19, SAOS2, and SJSA1 respectively. Quality control test was applied to the 3 gene expression datasets (Supplementary Table S1).

The gene expression profile was analyzed by EdgeR method DEGs were identified comparing the OS cell lines with osteoblast cells. Then, DEGs were selected considering: logFC ≥ 2, p-value ≤ 0.05, and FDR ≤ 0.05[21,22,33]. In this sense, 1,277 genes were identified as DEGs in SAOS2, among which 577 genes (45.2%) were up-regulated while the down-regulated genes were 700 (54.8%) (Fig. 1A). As well as, in SJSA1 were found 1,164 DEGs; 680 (58.4%) up-regulated and 484 (41.6%) down-regulated (Fig. 1A; Supplementary Table S2). Also, volcano and Heatmap plots were established to plot the DEGs of osteosarcoma cell lines (Fig. 1A and B).

DEGs, pathways-metabolism relationship, and PPI network.

To determinate the biological role of DEGs in osteosarcoma, a KEGG gen functional enrichment analysis was performed. A total of 112 and 70 KEGG pathways enriched down-regulated and up-regulated DEGs respectively in SAOS2, while in SJSA1, 81 were found down-regulated and 100 up-regulated. The KEGG enriched pathways were displayed in supplementary Table 2. A p-value ≤0.05 were considered as significative enrichment. The pathway enrichment analysis revealed that many DEGs were primarily involved with ECM-receptor interaction, focal adhesion, proteoglycans in cancer, small cell lung cancer, and adherent junctions (Supplementary Table S3).

The pathways found were filtered by CancerSEA considering the 107 pathways associated with metastasis, invasion, and EMT (45, 34, and 28 respectively). The KEGG pathways

related to these cancer characteristics were plotted (Fig. 2A-B - left panel). This analysis confirmed functional pathways related to focal adhesion, ECM-receptor interaction, regulation of cytoskeleton, cell adhesion molecules, proteoglycans in cancer, and adherent junctions (Fig. 2A-B - left panel). Interestingly all these pathways have been reported for invasion and metastasis in osteosarcoma [34–36]. Further, the DEGs associated the cancer pathways identified were reanalyzed with a GO-CC enrichment. Moreover, the GO-CC enrichment revealed the participation of both down and up-regulated DEGs in tight junction, cell-cell junction, focal adhesion, actin cytoeskelenton and filament, filipodium, and lamellipodium (Fig. 2A-B - middle panel).

Considering the flow of genetic information, we mapped the putative proteins encoded by DEGs associated with cancer pathways in STRING database. A PPI network describes the physical interactions between proteins, which indicates the number of connections (edges) that inside in a particular node (protein) or degree. Also, high connectivity (HC) in a node indicates that the node has direct interaction with many other distinct nodes. Proteins with HC are considered essential hubs of the network and it can be inferred which key biological processes are functionally connected.

In the present study, we generated four PPI networks from SAOS2 and SJSA1's DEGs up and down-regulated, considering a high confidence score (≥0.7) to eliminate PPIs with low probability/significance. Network parameters were evaluated with igraph R package. Various parameters of the PPI networks such as clustering coefficient, average degree, and hub value are shown in Table 2. Degree was taken as a parameter to visualize the network. (fig. 2A-B – right panel).

Hub analysis indicates that Interleukin-6 (IL6), Signal transducer and activator of transcription 1-alpha/beta (STAT1), CD44 antigen (CD44), and Interleukin-8 (CXCL8) were some of the best-ranked nodes in down-regulated PPI networks (fig 2A – right panel; Table 2; Fig. S1). While in the upregulated PPI networks proteins like MMP-2, Integrin beta-3, 4, and 5 (ITGB3-4 and 5), Collagen alpha-1 chain (COL1A1), and Decorin (DCN) were identified as hubs (Fig. 2B – right panel; Table 2; Fig. S1).

Functional expression of MMP-2 in osteosarcoma cell lines

The functional expression of MMP-2 was of our particular interest because it was found as hub in the networks. This protein has been related with cell invasion of some types of cancer, and even the synergism with activated ion channels such as Na_Vs [6,12,37–40]. Additionally, we wonder whether differentially expressed MMP-2 has a function in osteosarcoma cell lines. Therefore a gelatin zymography assay was performed to analyse the proteolytic activity of MMP-2 in supernatants of hFOB1.19, SAOS2, and SJSA1 cell cultures. The cells were incubated for 40 h with a medium with 1% FBS, the protein concentration of the supernatant was measured and the MMPs activity was evaluated. Thus, the activity of two MMPs (~72 and ~95 KDa) corresponding to the pro and active forms of MMP-2 and MMP-9 were revealed (Fig. 3).

For negative control the media (without cells) with 1% FBS was used, the basal activity of MMPs from FBS corresponding to ~72, and ~95 KDa was observed (line 1, Fig. 3). While the cell cultures treated with 100 ng/ml of PDB were used for positive control and the intensity of ~72 and ~95 KDa increased (line 2, Fig. 3). When osteosarcoma cells were incubated for 40 h with 1% FBS no difference was observed in the intensity of the ~95 KDa band, concerning the negative control. However, in the ~72KDa form that corresponds to MMP-2, the intensity was increased respect to negative control (line 3, Fig. 3). These results are according with the transcript found in the DEGs experiments and demonstrated that MMP-2 is expressed and functional at protein level in osteosarcoma cell lines.

Functional expression of Na_Vs in osteosarcoma cell lines

In order to know if synergism related with Na_Vs are also expressed in osteosarcoma, we used isoform-specific primers to amplify the different subtypes of Na_Vs alpha and beta subunits (Table 1) by RT-qPCR. For this validation, total RNA isolated from hFOB1.19, SAOS2, and SJSA1 were used to generate the cDNA library. To compare transcription levels PSMB2 housekeeping gene was used as a reference. The Ct was employed to determine the RNA quantity. Briefly, Ct is inversely proportional (on a logarithmic scale) to RNA quantity, thus lower Ct values correspond to higher RNA target amount.

The Ct values were the input to know the relative mRNA expression by the $2^{-\Delta\Delta Ct}$ method (Fig. 4A-B). This analysis confirms that there is no differential mRNA expression of the alpha and

beta Na_Vs genes in SAOS2, while in SJSA1 the $Na_V1.7$ gene is 5 fold over-expressed (Fig. 4C). Additionally, the over-expression of β1a, β1c, and β2 (2.63, 5.41, and 12.21 folds respectively) were identified (Fig. 4D). The relative mRNA expression of Na_Vs were consistent with RNA-seq results (Fig. S2), which, coincidentally, these values also agree with the published where deregulated expression of different Na_vs in cancer tissues has been associated with metastatic progression and cancer-related death [41]. To investigate if the up-regulation of the mRNA that encodes the Na_Vs channels have a functional contribution to the total sodium current of osteosarcoma cells, we performed a whole-cell patch clamp experiments in hFOB1.19, SAOS2, and SJSA1.

Interestingly in SAOS2 were not identified sodium currents (I_{Na}), while in hFOB1.19 and SJSA1 the characteristic I_{Na} were registered (Fig. 5). Although particularly making the whole-cell patch clamp to detect currents in flat cells represents some degree of difficulty, in hFOB1.19 and SJSA1 were successfully achieved, nevertheless did not show currents in SAOS2. Additionally, in presence of 1μM of TTX these currents were blockaded (Fig. 6). Confirming the functional expression of Na_Vs alpha subunits in osteosarcoma cell lines. Moreover is possible suggest that the currents are generated by $Na_V1.3$ and $Na_V1.7$ in hFOB1.19 and SJSA1 respectively. Also, with the electrophysiologycal characterization potassium currents (I_K) were recorded in the three cell lines (Fig. S3).

Participation of MMP-2 and Na_Vs in migration and invasion

Until now, the expression of $Na_V1.7$ at mRNA level has been confirmed, also electrophysiology characterization suggests the functional expression of Na_Vs TTX-S in hFOB1.19 and SJSA1. Hence, we explored the possibility that Na_Vs, MMPs, and NHEs and cathepsins activity could be involved in cell migration and invasion (Fig. S2) through *in vitro* migration and invasion assays.

The result of these tests allowed to identify a greater number of migratory and invasive SJSA1 cells (178 ± 21.39 and 56 ± 9.66 respectively), while SAOS2 presented a smaller amount (66.67 ± 19.4 and 20.89 ± 1.35 respectively); data that we expected considering reports of migration and invasion of these cell lines [42]. Also, hFOB1.19 was the least migratory and invasive cell line with 39.11 ± 8.46 and 7.78 ± 1.84 cells counted respectively,

Additionally, the cell migration and invasion assays were evaluated with or without blockers of Na_Vs (TTX 1μM), and K_Vs (TEA 20mM); NHE specific inhibitor (EIPA 10μM); protease inhibitors for MMPs (GM6001 25μM), and Cathepsins (E-64 100μM).

The osteoblast cell line (hFOB1.19) was used as a control, as we expected, it presents the fewest migratory cells, and they are reduced in invasion assay. Interestingly, in the presence of the different compounds, the migratory cells significative decreased. Whereas a difference in invasive cells is not observed (Fig. 6A-B). SAOS2 has been reported as a cell line with medium migration and invasion capabilities [42], in this sense, SAOS2 showed ~37% less migratory and invasive cells than SJSA1. Also, it was observed a diminution of migratory cells in the presence of GM6001 and EIPA, however, it was not so with E64, TEA, and TTX. In the invasion assay TEA and E64 decreased the invasiveness capacity while it is not observed difference with TTX and EIPA(Fig. 6A-B).

In the migration and invasion assays SJSA1 showed the highest migratory and invasive cell numbers, this is agreed with previous reports [42]. Interestingly, in this cell line was observed a significative diminution (>60%) in the number of migratory and invasive cells in the presence of the different blockers and inhibitors, suggesting the contribution of Na_Vs, K_Vs, NHE, Cathepsins, and MMPs in migration and invasion in osteosarcoma cell lines (Fig. 6A-B). These results were the base to evaluate the contribution of biomarkers proposed in migration and invasion.

Discussion

Children and adolescents patients diagnosed in advance stages of osteosarcoma manifest a highly aggressive cancer due a metastatic process. Metastasis is the principal cause of osteosarcoma-related deaths with a high mortality rate (20%-5-year survival rate). Metastasis is a complex and multi-step process that requires the acquisition of motile-migratory phenotype. In this sense, permits the adaptation of cancer cells to their microenvironment, emulating the activation of embryonic cells during development. The acquisition of this phenotype facilities the remodeling of cell-cell and cell-ECM adhesion to gain migratory abilities and thus invade adjacent tissues. This process involves a significant reorganization of the cytoskeleton, and the formation of actin-rich membrane protuberances (lamellipodia, filipodia, and invadopodia), key process for migration and invasion through the ECM. [5,43]. All these facts are important given that origin of osteosarcoma cells and are considered to arise from mesenchymal cells, several studies have revealed that EMT occurs in osteosarcoma and contributes to its initiation, progression, and metastasis (Zheng et al., 2021). Recently have been published studied of the molecular mechanism of osteosarcoma however it is still poorly understood. Therefore, it is crucial to identify new molecular targets as well as novel therapeutic strategies for the diagnosis, treatment, and prognosis of osteosarcoma.

In this study, we identified DEGs (1,373 up-regulated and 1,403 down-regulated in SAOS2; 1,350 up-regulated and 1,068 down-regulated in SJSA1) between osteoblast (hFOB1.19) and osteosarcoma (SAOS2 and SJSA1) cell lines. Moreover, enrichment analysis was performed using the KEGG database to determine the biological role of DEGs. The pathways identified were filtered with cancerSEA considering metastasis, invasion, and EMT. Interestingly, KEGG pathway enrichment analysis suggested that focal adhesion, ECM-receptor interaction, regulation of the cytoskeleton, cell adhesion molecules, and proteoglycans in cancer were significantly enriched. Also, the GO-CC enrichment analysis showed that cellular components including cell-cell junction, focal adhesion, actin filament, and lamellipodium were significantly enriched in osteosarcoma cell lines. Interestingly, these pathways and GO-CC terms have been previously reported with osteosarcoma differential expression analysis, and have been closely associated with metastatic behavior [44–46]. In addition to this, genes like SRC, STAT1, IL6, FN1, CD44, IL1B, MMP-2, ITGB3, ITGB4, COL1A1, and DCN were identified as hubs in the down and up-regulated PPI-network, which have participation in pathways related to

metastasis, invasion, migration, and EMT; and have already been previously reported as hubs in osteosarcoma [35,45,47–50].

Considering the flow of genetic information, it is possible to suppose that DEGs may also be expressed at the protein level. In this sense, PosthumaDeBoer et al (2013) reported a comparative proteomic analysis of cell surface proteomes of five osteosarcoma cell lines and three osteoblasts, including SAOS2. Among the proteins they found over expressed were CD44, COL1A1, COL6A1, FN1, ITGB4, ITGA2, ITGA6, MMP-2, PDGFRB, PRKCA, SDC1, SRC, STAT1, TGFB1, and TP53[51]. As well as, Jerez et al (2017) compared the secreted proteins and exosomes from SAOS2, MG63, and HOS with hFOB1.19. Interestingly, in SAOS2 were expressed proteins like CD44, MMP-2, PRKCA, SRC, STAT1, and TGFB1[52]. In this study, we found the genes that code for these proteins as DEGs and hubs.

MMPs are proteolytic enzymes implicated in different types of cancers, such as melanoma, breast cancer and osteosarcoma. MMPs secreted by osteosarcoma cells lines comprises a group of gelatinases, i.e., MMP-2 and 9, members are zinc-dependent endopeptidases. Gelatinases cleave gelatin (denatured collagen), a few types of collagen (including type IV), fibronectin, and all structural components of ECM (proteoglycans, elastin, laminin, etc) [17,53]. The MMPs family, which have been linked to ECM remodeling by the degradation have essential roles in diverse physiological processes like embryonic development, morphogenesis, tissue maintenance, and pathophysiological like rheumatoid arthritis, osteoporosis, and cancer metastasis [40,54,55]. Hence, MMPs could be interesting biomarkers for diseases related to the alteration of the ECM.

MMPs up-regulation in cancer promotes the degradation of the extracellular matrix, detachment, migration, and invasion and causes the cancer metastasis, and has been correlated with tumor aggression, metastasis, and poor prognosis [39,56–58]. For example, MMP-2 and -9 have been found over-expressed in highly aggressive pediatric sarcomas [59,60]. Also, several osteosarcoma cell lines have been shown the up-regulation of different MMPs, these proteins have participation in cell proliferation, migration, invasion, and metastasis, and blocking MMPs inhibits these cancer hallmarks [61–63]. In addition, the over-expression of MMP-2 was associated with poor prognosis and pulmonary metastasis in patients with osteosarcoma [39,56,57].

In the present study, the functional expression of MMPs was evaluated in hFOB1.19, SAOS2, and SJSA1 cell lines. The invasiveness of the cell lines was evaluated by zymography assay which permitted the investigation of the activity of the matrix gelatinases like MMP-2 and -9. Our analyses revealed the functional expression of MMP-2 in all cell lines. Interestingly, the highest amount of MMP-2 corresponds to cancerous cells (SAOS2 and SJSA1). The molecular heigh corresponded to pro and active forms (72 and 66 KDa respectively). These results corresponded with previous reports which have found the expression of MMP-2 at mRNA and protein level in several osteosarcoma cell lines, including SAOS2 and SJSA1 [62–65].

Also, functional expression of MMPs corresponding with a molecular weight of 92 and 83 KDa respectively was identified in the three cell lines, suggesting the presence of pro and active MMP-9. However, this enzyme was not found differentially expressed with RNA-seq and in the zymography was not observed as an important difference.

Furthermore, this is the first work that reports the functional expression of MMP-2 and -9 in human fetal osteoblastic cells (hFOB1.19). It was of our particular interest to find the functional expression of MMP-2 because several works have reported the participation of this protein in cell migration and invasion in some types of cancer, and even in collaboration with other proteins such as Na_Vs. [6,12,37,38].

The Na_Vs family is conformed by nine members ($Na_V1.1$ to $Na_V1.9$), all of them presents >50% aminoacid identity. Also, Na_Vs have a binding site to TTX, which permits the Na_V classification in TTX-S ($Na_V1.1$, $Na_V1.2$, $Na_V1.3$, $Na_V1.4$, $Na_V1.6$ and $Na_V1.7$), and TTX-R ($Na_V1.5$, $Na_V1.8$ and $Na_V1.9$) [66]. Some works have proposed the participation of different Na_Vs subunits in different cancer types and hallmarks like invasion and metastasis [38,67,68]. For example, Diss et al in 2005 reported the upregulation of $Na_V1.7$ in biopses of Pca, and the level expression was associated with strong metastatic potential [69]. Also, VGSC currents were recorded in gastric cancer cells, and was probed that the currents corresponded to $Na_V1.7$ [14]. The participation of $Na_V1.7$ could be through different vias, one of them is the activation of molecules that participate in cell motility like cAMP-dependent protein kinase catalytic (PK), ankyrins, troponins, and gelsolins which can be stimulated by Na_Vs activity. Thus the organization of the cytoskeleton is modified and cell migration could be enhanced. Considering the evolutionary relationship between these channels and the similarity of their

amino acid sequences is possible that their action mechanism and interactions could be similar in cancer, more specifically in migration and invasion.

The Ca^{2+} and pH are another way where Na_Vs could be involved in migration and invasiveness. In this sense Na^+ inward by Na_Vs activity can influence local Ca^{2+} through Na^+/Ca^{2+} exchangers (NCX). In turn, intracellular Ca^{2+} changes can affect the activities of proteins that modify actin polymerization like gelsolin (Ca^{2+} sensitive). Also, is possible that NHE7 activity would be modulated by $Na_V1.7$, the activity of NHE7 generates the optimal pH for the activation and function enhances of MMPs, cathepsins and integrins [9,10,12,68,70–72]

Similarly, K^+ channels are the largest and most diverse family of ion channels with more than 70 members reported in mammals. Their role in cell migration has been widely studied in cancer. For instance, $K_V10.1$ and $K_V11.1$ are regulators of tumor cell proliferation and migration, and upregulated expression is negatively correlated with patient prognosis. Has been suggested that $K_V11.1$ interacts with β-integrins by conformational coupling, and this interaction permits that both proteins reciprocally activate each other. Also, functional $K_V11.1$ channels strongly affect neurite outgrowth in neuroblastoma cells and cell migration by controlling cell-ECM interactions [73–75]. Wu in 2013 found $K_V10.1$ expressed in SAOS2, the results of migration assays confirm the participation of K_Vs in cell migration in SAOS2. Likewise, in SJSA1 the KCNS3 was identified as over-expressed and the treatment with TEA decrease the migratory and invasive cells.

In this study, we recorded Na_Vs currents TTX-S at the plasma membrane of hFOB1.19 and SJSA1 while in SAOS2 were not found. In this sense, it was in our interest to find up-regulated the $Na_V1.7$ gene in SJSA1. This suggests the functional expression of TTX-S channels in hFOB1.19 and SJSA1 but not in SAOS2, consistent with RNA-seq and RT-qPCR results, where the Na_Vs genes were not identified expressed in SAOS2. Also, K^+ currents were identified in the three cell lines; in SJSA1 these currents could be due to KCNB1 ($K_V2.1$) and KCNS3 ($K_V9.3$), which are up-regulated. Whereas the KCNH1 ($K_V10.1$) is up-regulated in SAOS2. Besides, in both SAOS2 and SJSA1 are down-regulated KCNQ3 ($K_V7.1$), KCNQ5 ($K_V7.5$), and KCNMA1 (Slo1). Nevertheless, it is necessary a more detailed characterization of K^+ channels and their currents.

To elucidate the role of Na_Vs and the molecules suggested in osteosarcoma metastasis, *in vitro* migration and invasion assays were performed with or without different inhibitors/blockers of Na_Vs. (TTX 1μM), NHE (EIPA 10μM), MMPs (GM6001 25μM), and cathepsins (E-64 100μM). These assays showed that hFOB1.19 is the cell line with the least ability to migrate and invade. In the presence of the inhibitors and blockers a significative difference in migration capacities was observed. Conversely, a significant diminution of the number of cells was observed only in the presence of TTX. It should be noted that this would be the first report to characterize the migratory and invasive properties of this osteoblast cell line.

SAOS2 presents medium capacities of migration and invasion characteristics. Interestingly, a significant decrease of migratory and invasive cells was observed in presence of EIPA, and TEA decreased the invasive cell capacities. Surprisingly it did not show a difference in migration and invasion in the presence of TTX. These results are consistent with mRNA expression and electrophysiological characterization, and suggest a different mechanism involve the participation of NHE and K_Vs in migration and invasion instead of TTX sensitive proteins in this cell line. Besides, SJSA1 was the cell line with the highest migratory and invasive capacities, and in presence of the inhibitors and blockers, the number of cells decreased significantly, suggesting the participation of $Na_V1.7$, NHE7 MMP2, and Cathepsins in SJSA1, a highly aggressive osteosarcoma cell line.

After all, with results obtained with RNA-seq, RT-qPCR, PPI networks, electrophysiology characterization, and *in vitro* migration and invasion assays, we propose a putative migration and invasion mechanism that could be related to osteosarcoma metastatic behavior. Which considers the differences between migratory and invasive capacities of cell lines used in this work (Fig. 8).

Conclusion

In this study we reported the identification of DEGs and pathways related to invasion, EMT and metastasis in osteosarcoma cell lines. The principally pathways identified were focal adhesion, ECM-receptor interaction, regulation of cytoskeleton, cell adhesion molecules, proteoglycans in cancer, and adherent junctions. Further, the GO-CC enrichment revealed the participation of both down and up-regulated DEGs in tight junction, cell-cell junction, focal adhesion, actin filament, and lamellipodium. Also, hub genes were identified by PPI network analysis, these genes could be proposed as biomarkers considering their importance in the osteosarcoma networks. Some interesting genes are COL1A1, CTNNB1, ITGB4, ITGB5, PRKCA, TGFB1, and MMP-2.

We reported the functional expression of MMP-2 in osteosarcoma cell lines, and the most invasive cell line secreted the highest amount of MMP-2. Furthermore, this is the first work that reports the functional expression of MMP-2 and -9 in human fetal osteoblastic cells (hFOB1.19).

Also, K^+ currents were recorded in the three cell lines, and the migratory and invasiveness capacities decreased in the presence of TEA. However, more studies are needed to understand the role of K_Vs in osteosarcoma cell migration and invasion. Similarly, Na_Vs currents were recorded in hFOB1.19 and SJSA1, in this work is proposed the participation of Na_Vs TTX-S (with a particular interest in $Na_V1.7$) in migration and invasion in SJSA1, the highest aggressive cell line. In SAOS2, Na_Vs are not identified at mRNA level nor in the cell membrane by patch-clamp. Finally, participation of Na_Vs, K_Vs, NHE, cathepsins, and MMPs were demonstrated in migration and invasion cells assays by the treatment with specific blockers and inhibitors.

Gene expression analysis provides invaluable opportunities to identify mechanisms responsible for the development of complex diseases like cancer. In this work, we could identify biomarkers that are related to invasion and metastasis and provided insights into the knowledge of osteosarcoma pathogenesis.

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