Research Article

Acquiring Metastatic Competence by Oral Squamous Cell Carcinoma Cells Is Associated with Differential Expression of α-Tubulin Isoforms

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Received 7 February 2012; Accepted 17 April 2012

Academic Editor: M. Roach

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We performed comparative global proteomics analyses of patient-matched primary (686Tu) and metastatic (686Ln) OSCC cells. The metastatic OSCC 686Ln cells showed greater in vitro migratory/invasive potential and distinct cell shape from their parental primary 686Tu cells. Ettan DIGE analysis revealed 1316 proteins spots in both cell lines with >85% to be quantitatively similar (<2 folds) between the two cell lines. However, two protein spots among four serial spots were highly dominant in 686Ln cells. Mass spectrometry sequencing demonstrated all four spots to be α-tubulin isotypes. Further analysis showed no significant quantitative difference in the α-tubulin between the two cell lines either at mRNA or protein levels. Thus, two distinct isoforms of α-tubulin, probably due to posttranslational modification, were associated with metastatic 686Ln cells. Immunofluorescence demonstrated remarkable differences in the cytosolic α-tubulin distribution patterns between the two cells. In 686Tu cells, α-tubulin proteins formed a normal network composed of filaments. In contrast, α-tubulin in 686Ln cells exhibited only partial cytoskeletal distribution with the majority of the protein diffusely distributed within the cytosol. Since α-tubulin is critical for cell shape and mobility, our finding suggests a role of α-tubulin isoforms in acquisition of metastatic phenotype and represents potential target for therapeutic intervention.

1. Introduction

Oropharyngeal squamous cell carcinoma (OSCC) ranks among the top ten most frequent cancers worldwide. Despite recent advances in cancer treatments, the 5-year survival rate for OSCC patients has not changed significantly over the past 30 years, remaining at 30–50%. Most patients with OSCC have metastatic disease at the time of diagnosis, and unlike other malignancies, OSCC spreads exclusively via lymphatic routes to the cervical nodes [1]. The 5-year survival rate is <50% even in patients with a single unilateral lymph node metastasis [1]. Therefore, cervical lymph node metastasis is the major determinant of survival in patients with OSCC. Metastasis which is defined as the spread of tumor cells from the primary site to other tissues of the body is a complex process, involving invasion into adjacent tissues, intravasation, arrest within the microcirculation, and establishment of new growth in regional lymph nodes and/or distant organs [2–4]. To acquire these properties, tumor cells undergo selective changes in their gene expression patterns, leading to overexpression of metastasis-promoting molecules [5, 6]. Recent gene expression profiling studies have demonstrated that cancer’s natural history, including metastasis and response to treatment, is indeed encoded in the primary tumors [6–8]. Furthermore, these studies have documented that specific gene expression patterns of primary tumors are better than the routine clinic-pathologic indicators in predicting metastasis, recurrence, and the patient’s overall survival.

Tumor cells acquiring metastatic phenotype exhibit specific molecular alterations that regulate cell motility and adhesion, the epithelial to mesenchymal transition (EMT),
and extracellular matrix remodeling. Because development regional lymph node metastasis causes much of the morbidity and incurability of OSCC, there is a critical need to identify molecules that confer metastatic phenotype in OSCC [1]. Although there are various methods that have been used to identify molecules involved in metastasis, one of the most convincing approaches is a comparative proteomic analyzes of patient-matched primary and metastatic tumor cells. Identification-metastasis-associated proteomic aberrations are critical for the development of new diagnostic tests for prediction and detection of OSCC metastasis and formulate treatment strategies for OSCC metastasis.

Previously, we reported the gene expression signature that distinguishes primary and metastatic tumor cells derived from the same patient [9, 10]. Here, we extend our study to characterize the proteomic signature of metastatic OSCC cells. We identified a qualitative and qualitative difference in the expression pattern of α-tubulin isofoms in metastasizing cells which correlates with their increased motility in vitro.

2. Materials and Methods

2.1. Cell Lines. Details related to primary OSCC (MDA686Tu) and metastatic OSCC (MDA686Ln) cell lines have been described previously [9]. Briefly, the MDA686Tu (686Tu) and MDA686Ln (686Ln) cell lines were established simultaneously from the primary tumor and lymph node metastasis of OSCC (T3N3B) involving the left base of the tongue of a 49-year-old male. These cell lines were maintained in DMEM/F12 50/50 mix (Cambrex) containing 10% fetal bovine serum (FBS), 0.4 m urea, 2 M thiourea, 2% CHAPS, 50 mM DTT and 1% Phamalyte (pH3–10NL). The first-dimension IEF on a rod gel (12 cm × 1.0 mm, pH3–10) was performed under a programmed gradient 200 V to 1,500 V over 18 hrs (total 25 kVh). The first-dimensional gel was equilibrated in 1x SDS-PAGE loading buffer for 3 hrs and loaded onto an isocratic slab SDS-PAGE gel (8–15%) for electrophoresis under constant current of 20 mA overnight. The SDS-PAGE gels were imaged by sequential fluorescence emission spectrosopy using a Typhoon 9400 laser scanner (GE Healthcare, Piscataway, NJ) for each of the spectrally resolvable Cy3/5 dyes (Cy3, 532 nm/580 [bp ± 15] nm; Cy5, 633 nm/670 [bp ± 15]) and digital images recorded using ImageQuant (GE Healthcare) software. Individual protein spots observed within each of the 686Ln (Cy3) or 686Tu (Cy5) images were then quantified and compared after volumetric rendering using the differential in-gel analysis (DIA) algorithm within DeCyder (v5.5; GE Healthcare) software. Individual spots of interest that exhibited a statistically significant difference in volume between the two cell lines were identified. In a conventional 2D electrophoreses under the same conditions, the interested spots were picked after Cooomassie blue staining for further mass spectrometry. Mass spectral analyses were performed in the Translational Proteomics Core at UTHHealth. Briefly, the proteins were in-gel digested as previously described (http://msf.ucsf.edu/ingel.html) [11]. The tryptic digests were taken to dryness in a Thermo SpeedVac and dissolved in 20 μL of 2% acetonitrile, 0.1% formic acid (solvent A). Aliquots of the digest were analyzed by LC/MS/MS on an Agilent 6538 UHD Accurate-Mass Quadrupole Time-of-Flight (Q-TOF) mass spectrometer equipped with an Agilent 1260 nano-LC system. The reverse phase chromatography was performed on Agilent High Capacity Chip (143 mm) using solvent A as initial mobile phase and varying percentages of solvent B (90% acetonitrile, 0.1% formic acid) to constitute a five-stage gradient elution (5–30% B for 28 mins; 30–40% B for 2 mins; 40–90% B for 2 mins; 90–3% B for 2 min). Electrospray ionization was operated at the spray voltage of 1.75 kV. Mass spectral data was extracted with MassHunter Quantitative Analysis package and peptides were identified from MSMS spectra with MASCOT. The MASCOT search was performed with a peptide tolerance of 5 ppm and an MSMS tolerance.
of 0.05 Da, fixed modification was carbamidomethyl and variable methionine oxidation. Identification of nontryptic fragments was performed manually with an initial search on the basis of predicted peptide masses of all possible fragments resulting from novel cleavage sites in the hinge region. The MSMS spectra of suspect peaks were verified by manual de novo peptide sequencing to confirm their identities.

2.4. Immunofluorescence. Cell suspensions were seeded to each well of 8-well Chamber slides (Fisher Scientific, Rochester, NY) for overnight at 37°C, 5% CO2. The cells were fixed and permeabilized with a kit (Cytofix/Cytoperm, BD, San Jose, CA). After thorough wash with cold PBS, the cells were first incubated with anti-CD32 antibody and normal goat serum for prevention of nonspecific binding of mouse or goat IgG. The cells were then incubated with mouse monoclonal antibody to α-actin (clone AC-40, Sigma, St. Louis, MO) or β-actin (clone AC-1, Sigma, St. Louis, MO), followed incubation with FITC-labeled goat anti-mouse IgG (Southern Biotech, Birmingham, AL). The cells were further stained by with Alex(R) 555-labelled rabbit monoclonal antibody against α-tubulin antibody (Cell Signaling Technology, Beverly, MA). Finally, the cells were counter stained with DAPI. The cells were observed under a fluorescent microscope (Eclipse 80i, Nikon, Tokyo, Japan). The digital images were recorded and analyzed by NIS-Elements (Nikon Image System).

2.5. Statistical Analysis. Two-sample t-test and one-way ANOVA were used to determine the statistical significance of the differences in migration and real-time PCR between 686Ln and 686Tu lines. Statistical analyses for DIGE were performed using ImageQuant software from GE Health.

3. Results and Discussion

3.1. Higher Invasion Potential of 686Ln Cells Isolated from Regional Lymph Nodes. Previously, we analyzed the global gene expression profiles of 686Tu and 686Ln and demonstrated that expression levels of >90% of the genes in cell lines derived from the primary and metastatic tumors of the same patient were identical to each other than the two metastatic cell lines from two different patients [9]. In line with the published reports, our study confirmed that the gene expression pattern of primary tumor cells are mostly preserved in their metastatic counterpart except for a few differentially expressed genes which are implicated in promoting metastasis [9, 12]. We performed cell motility and invasion assays to characterize the phenotypic differences between 686Tu and Ln cells. In the cell motility assay performed using the cell culture inserts without Matrigel coating, 686Ln cells revealed enhanced migration which was 5.5 fold higher than that of 686Tu cells (left panel in Figure 1(b)). In the invasion assay performed using the cell culture inserts coated with three-dimensional Matrigel, invasion of both cell lines were greatly decreased. However, the reductions rates were significantly different between 686Tu and 686Ln cells. The 686Ln cells showed only 3-fold reduction as compared to 686Tu cells with 7.1-fold reduction (Figure 1(c)). As a result, only a 3-fold difference was observed between 686Ln and 686Tu cells when invading through Matrigel, as compared to 5.5-fold difference when migrating through noncoated membrane. This data suggests that the molecular machinery that underlies cell motility is more critical for metastatic phenotype than their extracellular matrix degrading molecules which are essential for invasion through the Matrigel. Tumor cells are known to change their phenotype during culture. However, our result showed that the metastatic 686Ln cells demonstrate higher motility and invasive potential than their parent primary tumor cells and indicate that the phenotypic features acquired in vivo are also preserved in cultured cells. Therefore, this pair of cell lines represents an ideal model to characterize proteomic signature that is causal for metastatic phenotype.

3.2. Qualitative and Quantitative Differences in α-Tubulin Expression Patterns between 686Tu and 686Ln Cells are Caused by Posttranslational Modifications. Proteins from the two cell
lines were used for DIGE 2D gel electrophoresis. The scanned Cy3 and Cy5 images for 686Ln and 686Tu, respectively, displayed similar spot distribution patterns (Figure 2(a)). In line with our gene expression profiling data, the metastatic OSCC cells and its parent primary tumor cells revealed almost identical cellular protein profiles in CyDy DIGE analysis (Figure 2(a)). Based on our previous testing, over 5-fold difference in a protein of two sources would generate obvious green or red spot. In combined image, dots with obvious green or red color were visible (Figure 2(b)). Squared area in Figure 1(b) is an example to show different colors. With $2 \times 10^4$ pixels as background, the software detected 1316 protein spots in combined image for 686Tu (Cy5) and 686Ln proteins (Cy3), see Figure 3. Pixel volume for each spot was calculated and compared between 686Ln and 686Tu cells using 686Tu as standard. Plot with log volume ratios against spot numbers fit well with Gaussian distribution ($R^2 = 0.9629$) with 0.032 $\pm$ 0.16 in Log scale or 1.033 $\pm$ 1.176 folds (Figure 3). To estimate how closely the two cell lines were related, we used 2-fold differences in pixel volume as threshold. Calculation demonstrated that 686Ln and 686Tu cells showed similar volumes in 1159 spots (88.1%), suggesting their close relationship. In addition, 686Ln cells showed increased volume in 70 spots (5.3%) and decreased volume in 87 spots (8.6%), as compared to those of 686Tu cells. However, most spots with different volumes between the two cells clustered between 2 to 4 folds (Figure 3).

In this study, we solely focused on the protein spots, which were significantly increased in 686Ln. Plot of log volume ratio versus max volume revealed two protein spots which showed more than 5-fold increase in 686Ln cells with volumes higher than $2 \times 10^7$ pixels (arrows in Figure 3). These two protein spots were identified to be two green spots in an acidic region (pH 5–5.5) with molecular weight 40–60 kD (squared area in Figure 1(b)). These two green spots were among series of 5 dots with similar molecular masses of 55 kD. The two green spots migrated to higher pH region (pH 5.5). Those 5 proteins were designated as number 1 to 5 with number 1 and 2 for the two green spots (Figure 3(a), arrows in Figure 3). Calculation of pixel volumes demonstrated that number 1 and 2 in 686Ln cells were 7.5 and 7.1 folds of those in 686Tu cells. On the other
hand, volume ratios between 686Ln and 686Tu cells were 1.02, 0.76, and 0.69 for spots number 3, 4 and 5, respectively. At least three spots in the adjacent area were found to be at certain pixel ranges. Related pink curve is actual distribution of spots versus their pixel volume ratio and green curve is normalized Gaussian distribution \( R^2 = 0.963 \). Arrows indicate two protein spots to be studied.

![Figure 3: Scattergram depiction of DIGE data to generate protein expression profile of 686Tu and 686Ln cells. Each dot represents a protein detected in 686Tu and 686Ln cells. The dots are expressed as bigger pixel volume in either cell (right Y-axis), and plotted against log scale of pixel volume ratio between 686Tu and 686Ln (X axis), with 686Tu as standard. Two vertical lines indicate two-fold difference limits between the two cells. Left Y-axis is number of spots at certain pixel ranges.](image)

Next, we examined whether the immunofluorescence staining patterns of α-tubulin protein in this pair of cell lines would reveal any remarkable quantitative and/or qualitative differences. Overall, 686Ln cells showed a higher intensity of anti-α-tubulin immunofluorescence staining compared to 686Tu cells (Figure 6). Both cell lines demonstrated a higher density of α-tubulin expression in their perinuclear region. A comparison of the staining patterns of α-tubulin in 686Tu and Ln cells showed significant qualitative differences in the α-tubulin filament distribution. In 686Tu cells, α-tubulin proteins formed a network composed of coarse filaments, probably associated with cytoskeleton. Pattern of this network was similar to those in many other cell types including nontransformed cells [13]. In contrast, α-tubulin in 686Ln cells exhibited only partial cytoskeletal distribution with majority the protein diffusely distributed within the cytosol. As a result, a uniform filament network as seen in 686Tu cells was not evident in 686Ln cells (Figure 6).

Examination of the cells under higher magnification revealed that the cytoskeleton of 686Ln cells is composed of a network of much finer α-tubulin filaments (Figure 6). It should be noted that the 686Tu and Ln cells exhibit distinguishable morphologies, especially after migrating through the barrier membrane during motility and invasion assays. Almost all 686Tu cells displayed a round-flattened shape (Figure 1(a)). In contrast, more than 80% of 686Ln cells were elongated and branched, with only a small fraction to be in round spread shape (Figure 1(a)). Hence, we asked whether the unusual α-tubulin network in 686Ln cells was due to overlapping of multiple layer of the network because of their elongated and rounded morphology. We selectively examined a number of 686Ln cells with flattened morphology and found that none of these cells exhibited α-tubulin staining pattern similar to that of 686Tu cells. This finding suggests that the observed difference in the α-tubulin networks between 686Ln and 686Tu cells was not an artefact related to the cellular morphology. Moreover, we compared the immunofluorescences staining patterns of α-actin and β-actin in these two cell lines and showed that their staining patterns are indistinguishable between 686Tu and 686Ln cells (Figure 6). The sizes of α- and β-actin filaments are similar between these two cell lines, except for minor differences in their distribution patterns. These findings strongly suggest that the altered α-tubulin filament distribution pattern in 686Ln cells is most likely due to its posttranslational modification resulting in altered polymerization and/or increased solubility.

Our proteome analysis confirms that two distinct isoforms of α-tubulin are found in higher quantity in 686Ln than in 686Tu whereas the other two α-tubulin isoforms are not. Moreover, there is no significant quantitative difference either in the α-tubulin transcripts number or total protein levels between these two cell lines. It raises an interesting
question whether these two distinct α-tubulin isoforms detected in 686Ln cells are unique for metastatic phenotype. Although our study does not provide direct evidence to support that the differences in the α-tubulin isoforms and distribution pattern in 686Ln cells are causative for metastasis, published reports have suggested a critical role for tubulin isoforms in tumor cells motility and metastasis.

Microtubules, a major component of cytoskeleton are involved in various cellular processes such as cell division, intracellular organelle trafficking, secretion, maintenance of cell shape, and motility. Tubulin (100 kDa of two subunits), a major component of microtubules, is a heterodimer of α- and β-tubulin isotypes which are translated from seven genes [14]. Differential expression of α- and β-tubulins isotypes are associated with tumorigenesis, metastasis, and resistant to chemotherapy [15–18]. Increased expression of class IV β-tubulin isotype in breast cancer cells confer them increased resistance to docetaxel [16]. Docetaxel and paclitaxel are antimitotic chemotherapy drugs used for treating metastatic breast cancer. These drugs bind to β-tubulin and halt cell division at metaphase by preventing spindle assembly [19]. In contrast to β-tubulin, α-tubulin isotypes undergo significant posttranslational modifications that determine microtubule assembly, distribution pattern, and cellular phenotype. Polyglutamylation and detyrosination of α-tubulin are frequently noted in prostate cancer cells but not in nontransformed prostate epithelial cells [20]. Increased expression of acetylated of α-tubulin is specific for aggressive hormone-refractory prostate cancer cells [17].

Although it is not clear whether tubulin is directly involved in metastatic process, recent studies suggest that abnormal assembly of tubulin monomers may be associated with tumorigenic and metastatic phenotypes [15–17, 21–25]. Several potential mechanisms have been proposed. Microtentacles formed by tubulins in breast cancer cells accelerated cell detachment from ECM, which was a necessary step for metastasis [26–28]. Active disassociation and re-assembling of tubulins promotes cancer cells motility [29].

It has been shown that the cell motility is modulated by α-tubulin acetylation/deacetylation by impacting the assembly and disassembly of microtubules [30, 31]. Although the enzyme responsible for α-tubulin acetylation is yet to be identified, deacetylation of α-tubulin is catalyzed by histone deacetylase 6 (HDAC6) [32]. On the other hand, HDAC6 inhibitor blocks tumor cells migration and reduces their metastatic potential by inducing acetylation of α-tubulin [33].
Our findings indicate that the morphologic changes exhibited by 686Ln cells during their in vitro migration/invasion are reminiscent of morphologic features associated with epithelial-mesenchymal transition (EMT) in which epithelial cells acquire fibroblast-like properties [34]. Epithelial malignant tumor cells are critically dependent on EMT for their invasion and metastatic spread [34]. Recently, it was reported that transforming growth factor-β1- (TGF-β1-) induced EMT in tumor cells is indeed mediated by deacetylation of α-tubulins by HDAC6 [35]. Previous studies on tubulin family have shown that members of α-tubulins have very similar molecule weight and isoelectric point, which make them difficult to be separated even in 2D electrophoresis. Moreover, both tubulin spots specific for
686Ln cells were characterized as α tubulin 1C. Based on the published reports and our findings, we propose that two isoforms of α tubulin in metastatic 686Ln are caused by posttranslational modification, most likely deacetylation. Diffuse distribution of α tubulin in 686Ln suggests relatively unstable microtubules or presence of soluble α tubulins due to posttranslational modifications [18, 36]. However, the biochemical features of these posttranslational modifications remain to be explored. Although the findings of this study are encouraging, the data are preliminary based on in vitro studies. Hence, this data needs to be confirmed in vivo by examining a larger number of patient matched primary and metastatic OSCC tumor sections.

4. Conclusion

In conclusion, metastatic OSCC cells exhibit increased in vitro motility and Matrigel invasiveness than their parental primary tumor cells derived from the same patient. We identified two distinct α-tubulin isoforms specific for metastatic cells that are not present in its parental primary tumor cells. Moreover, α-tubulin filaments exhibit distinctly different distribution pattern in metastatic cells compared to its parental primary tumor cells. We, therefore, propose that differential posttranslational modifications of α-tubulin are involved in acquiring metastatic phenotype in OSCC and characterization of these specific modifications may open new avenues for therapeutic intervention against OSCC metastasis. However, it remains to be determined whether the differential expression of metastatic phenotype-specific α-tubulin isoforms can be demonstrated in patient-matched primary and metastatic tumor specimens.

Conflict of Interests

The authors declare that there is no conflict of interests.

Authors’ Contribution

B. Lou initiated and performed basic experiments. D. Engler conducted image analysis in 2D Ettan DIGE. W. Dubinsky directed peptide sequencing. J. Wu provided technical support for cell culture and in vitro assays. N. Vigneswaran served as the principal investigator of this project and prepared the manuscript.

Acknowledgments

The authors would like to thank Mrs. Kathleen Hoch (University of Texas HSC at Houston) for her technical support for peptide sequencing and Kiana Parker (University of Texas HSC at Houston) for immunofluorescent imaging. They are thankful to Dr. Peter G. Sacks (Department of Basic Science and Craniofacial Biology, New York University College of Dentistry) for providing them the primary and metastatic OSCC cell lines MDA686Tu and MDA686Ln. This work was supported by NIH/NIDCR Grant R21DE019956 (to N. Vigneswaran).

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