

Original Article

Validation of esophageal squamous cell carcinoma candidate genes from high-throughput transcriptomic studies

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Abstract: In a recent study, a unique gene expression signature was observed when comparing esophageal squamous cell carcinoma (ESCC) epithelial cells to normal esophageal epithelial cells using laser capture microdissection (LCM) and cDNA microarray technology. To validate the expression of several intriguing genes from that study (KRT17, cornulin, CD44, and EpCAM), we employed two new technologies, expression microdissection (xMD) for high-throughput microdissection facilitating protein analysis and RNAscope for the evaluation of low abundant transcripts *in situ*. For protein measurements, xMD technology was utilized to specifically procure sufficient tumor and normal epithelium from frozen human tissue for immunoblot analysis of KRT17 (CK17) and cornulin. A novel *in situ* hybridization method (RNAscope) was used to determine the transcript level of two relatively low expressed genes, CD44 and EpCAM in both individual formalin-fixed paraffin-embedded (FFPE) tissue sections and in an ESCC tissue microarray (TMA). The results successfully confirmed the initial expression pattern observed for all four genes, potentially implicating them in the pathogenesis of ESCC. Additionally, the study provides important methodological information on the overall process of candidate gene validation.

Keywords: Expression microdissection, esophageal squamous cell carcinoma, RNAscope, immunoblot

Introduction

In spite of recent advancements in diagnostic methods and novel treatment options, esophageal cancer (EC) remains the sixth-leading cause of neoplasm-related death in the world [1]. Esophageal Squamous Cell Carcinoma (ESCC), the most common subtype, is typically diagnosed after the onset of symptoms and is therefore associated with poor prognosis [2]. Clearly, both the development of novel biomarkers for early detection and development of new therapeutic targets for treatment of advanced cancer are required to reduce esophageal cancer mortality.

Previously, our laboratory completed a study using an innovative tissue microdissection

strategy and cDNA microarrays to measure gene expression profiles associated with cell differentiation and tumorigenesis of ESCC cells [3]. This initial analysis identified important gene expression differences that indicated normal differentiated cells expressed a markedly different expression profile compared to normal basal epithelial cells and tumor. Interestingly, tumor and normal basal cells demonstrated more closely related expression profiles. The data showed that tumor contained 575 and 2990 differentially expressed genes at a >2-fold level when compared to normal esophageal basal cells and normal differentiated esophageal cells, respectively. Four genes of interest, including KRT17, cornulin, EpCAM and CD44, were chosen for validation in this study.

Keratin 17 (CK17 or KRT17) was one of the most significantly up-regulated transcripts in the tumor vs. normal comparison, with more than a 12-fold over-expression in esophageal cancer cells [3]. Conversely, CRNN (cornulin) was decreased in both the tumor vs. normal and tumor vs. basal cell comparisons, with a range of 76-127 fold down-regulation in tumor compared to normal epithelial cells [3]. Epithelial cell adhesion activating molecule (EpCAM), an epithelial transmembrane glycoprotein, is overexpressed in several malignant tumors [4] and demonstrated a 3.4-fold increased expression in ESCC cells than in normal epithelium [3]. Interestingly, CD44, a tumor initiating marker [5], did not demonstrate a significant differential expression pattern between tumor cells and normal epithelial cells, but showed higher expression in normal basal cells than normal differentiated cells [3].

In the present study, we utilized expression microdissection (xMD), a second-generation tissue microdissection technology [6, 7] to specifically procure cytokeratin-positive tumor cells as well as matched normal epithelial cells from six frozen ESCC cases to validate the expression of the KRT17 and cornulin genes at the protein level. We extracted the total protein from the microdissected tissue samples and performed immunoblot analysis. However, CD44 and EpCAM protein expression proved difficult to detect via immunoblot due to the lack of sensitivity inherent in the immunoblot assay. Thus we utilized a more sensitive RNA *in situ* hybridization method, RNAscope, to detect their transcript distribution in tumor and normal epithelial cells in individual formalin-fixed paraffin-embedded (FFPE) histological ESCC sections and in a FFPE tissue microarray (TMA) comprising of ESCC and normal esophageal tissue.

Materials and methods

Tissue specimens and TMA construction

Frozen tissue from six anonymized cases, including matched ESCC and normal esophageal tissue were obtained from Shanxi Cancer Hospital and Institute of China. The study was approved by the Institutional Review Boards of the collaborating institutions (Single Project Assurance Number #S-12118-01): Shanxi Cancer Hospital and Institute, Taiyuan, Shanxi

Province, China; and the National Cancer Institute, Bethesda, MD, USA. Samples were embedded in Optimal Cutting Temperature (OCT) media, then stored in -80°C freezer. Prior to immunohistochemical (IHC) staining, the tumor and normal tissue were sectioned at a 10 µm thickness using a Leica cryostat, placed on charged glass slides and kept in -80°C for up to one month. Three formalin-fixed paraffin-embedded (FFPE) anonymized tissue blocks containing both normal epithelium and ESCC tumor cells and two anonymized tissue microarrays (TMA) were obtained from the same hospital. The methodology of the TMA construction was as previously described [8]. Briefly, ESCC tumor tissues and normal epithelium tissues were sampled from 25 patient cases seen between 2005 and 2007 at the Shanxi Cancer Hospital. Tumor and normal tissue samples were arrayed into two separate TMA recipient blocks with 1.00 mm diameter cores. The cases used in the protein analysis and *in situ* hybridization studies were different, i.e. there were no recurring cases evaluated by both assays.

Immunohistochemistry

Frozen sections for each case were stained with an optimized IHC method for xMD. Briefly, sections were put in 70% ethanol for 5 minutes and rinsed in water. The slides were then incubated in peroxidase block (Invitrogen, Grand Island, NY) for 10 minutes, followed by washing in PBS for 5 minutes. Primary antibody cytokeratin AE1/AE3 (1:50, Diagnostic Biosystems, Pleasanton, CA) was added to the tissue section and incubated for 30 minutes at room temperature (RT), followed by washing in PBS for 5 minutes and secondary antibody (Dako, Carpinteria, CA) incubation for another 30 minutes. The staining solution of 3,3'-Diaminobenzidine (DAB) (Dako, Carpinteria, CA) was prepared according to the manufacturer's instruction. The DAB solution was incubated on the tissue sample for up to 5 minutes. After DAB incubation, the tissue sections were dehydrated sequentially in 70%, 95%, 100%, 100% ethanol for 1 minute for each step and finally soaked in xylenes for 1 minute. Dehydrated sections were stored in an airtight container with desiccant until xMD was performed. All steps were completed at room temperature fol-

lowing the initial removal of the tissue sections from the -80°C freezer.

Expression microdissection (xMD)

xMD was performed with two devices: a hand-held laser device, Quazar SDL15 laser diode gun (Biotechnique Avance, UK), with the following parameters: (120-240 V ~ 50-60 Hz, 100 W; laser wavelength 808 nm) and a broad-spectrum flashlamp (SensEpil, Home Skinnovations Inc, Canada). Generally, we used the laser gun at intensity setting 3 and pulse frequency setting 3 for 200-1000 laser pulses per section, depending on the staining intensity of the IHC. The flashlamp was used at energy level 2 for 5-6 flashes per section. For both systems, an ethylene-vinyl acetate (EVA) membrane (CoTran 9715 film, 3 M, St. Paul, MN) was placed on top of the section and a vacuum was applied to improve contact between the membrane and the tissue section. Once the dissection was complete, the EVA membrane was carefully removed from the section and placed into an airtight container with desiccant until all sections were dissected.

Protein elution and western-blot

To extract the proteins from the dissected cells, the EVA membranes were cut into pieces post-dissection and placed in a 1.5 ml tube containing 40-120 µl denaturing lysis buffer (Boston BioProducts, Ashland, MA), depending on the number of membranes. The tube was then incubated at 95°C for 10 minutes and sonicated 3 times for 10 seconds each time, allowing 1 minute interval incubation in ice between each sonication. The tube was then incubated at 37°C with 800 rpm shaking for 30 minutes. Finally the solution was centrifuged at 13,000 rpm for 10 minutes and the supernatant was moved to a new tube and stored at -20°C.

Protein concentration was assayed using a Micro BCA protein assay kit (Thermo Scientific, Rockford, IL) according to the manufacturer's instructions. Equal amounts (4-10 µg) of protein samples of tumor and matched normal tissue were electrophoresed in 4-20% Tris-Glycine gels (Invitrogen, Carlsbad, CA) at a constant voltage of 125 V for 1.5 hours. The proteins were then transferred to a nitrocellulose membrane via the iBlot 7-minute blotting system (Invitrogen, Carlsbad, CA) according to manu-

facturer's instructions. The immunoblot process was completed using the Protein Detector TMB Western Blotting Kit (KPL, Gaithersburg, MD) and primary antibodies (abcam, Cambridge, MA) with the following dilutions; rabbit-anti-cytokeratin 17 (1:800), rabbit-anti-cornulin (1:800), rabbit-anti-periostin (1:1000), rabbit-anti-CD44 (1:3500), rabbit-anti-EpCAM (1:2000) and mouse-anti-Actin (1:400). Targeted protein bands were visualized with LumiGLO Reserve Chemiluminescent Substrate (KPL, Gaithersburg, MD) and exposed to Kodak films for 1 second to 5 minutes according to the intensity of the protein bands (Carestream, Rochester, NY). For quantification, the intensity of each band was evaluated using Quantity One image analysis software (Bio-Rad, Hercules, CA) according to the manufacturer's instructions.

RNA in situ hybridization with RNAscope 2.0 FFPE assay

In situ hybridization for quantitative validation of the gene expression differences was performed using the RNAscope 2.0 (Brown) FFPE Assay (Advanced Cell Diagnostics, Inc., Hayward, CA) according to the manufacturer's instructions. Briefly, 5 µm-thick formalin-fixed, paraffin-embedded tissue sections were deparaffinized and pretreated with protease digestion prior to hybridization with a target probe. An HRP-based, temperature sensitive signal amplification system was then hybridized to the paired target probes followed by color development with DAB. Control probes for the bacterial gene DapB (negative control) and for the house-keeping gene POLR2A (positive control) were also included for each case. Three genes including KRT17, CD44 and EpCAM were selected for RNA level gene expression validation. Cornulin (CRNN) was excluded since there was no commercially available probe. Positive staining was identified as brown punctate dots present in the section. Cases were manually evaluated independently by authors Q.D. and W.Y. and scored based on the manufacturer's semi-quantitative scoring guideline. Paired t-tests were used to identify differences in matched tumor/normal gene expression.

Initially, three FFPE ESCC cases with adjacent normal regions were evaluated for RNA *in situ* hybridization analysis with the RNAscope 2.0 FFPE Assay as a pilot study. Once the perfor-

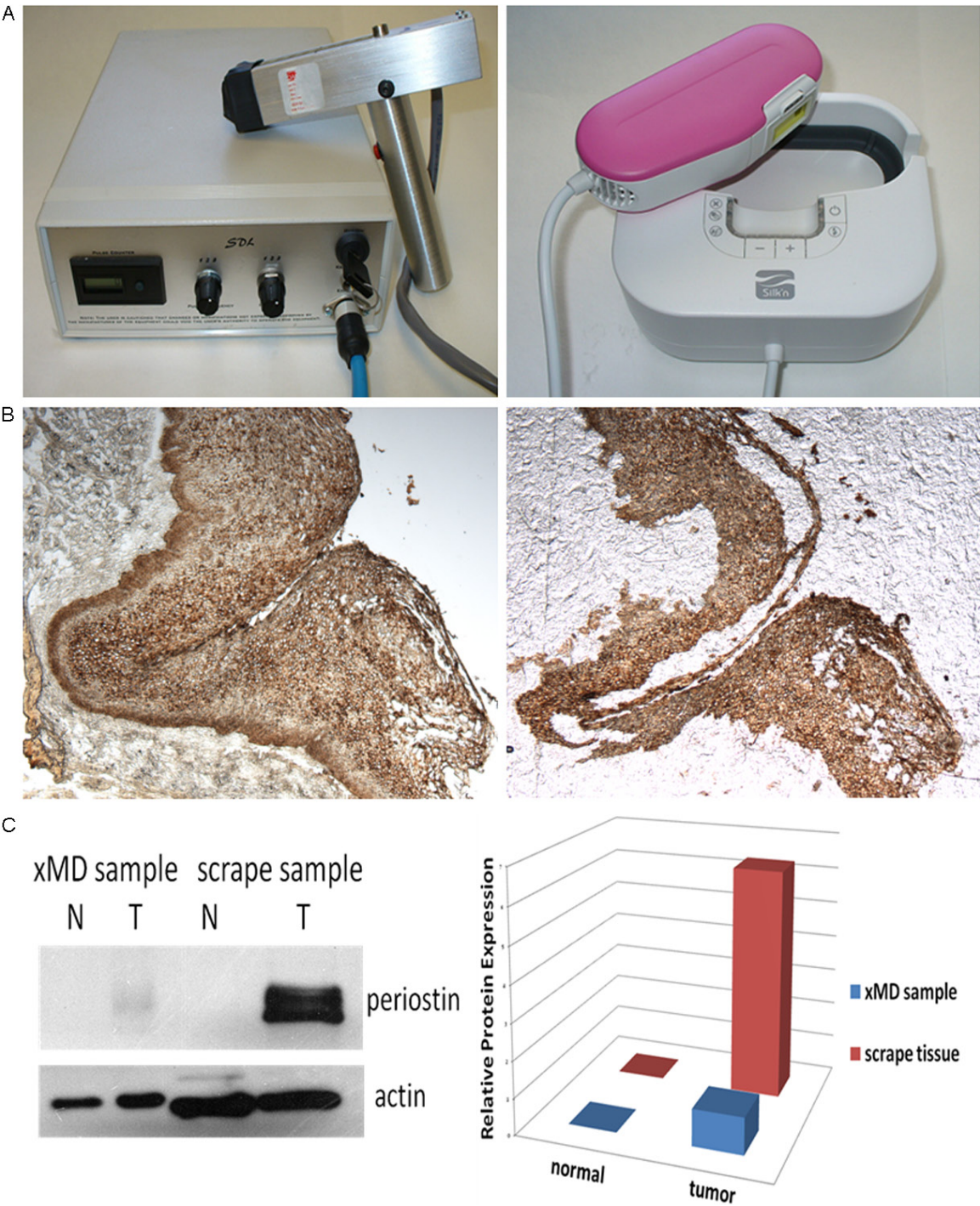


Figure 1. Evaluation of the efficiency and specificity of xMD. A: The two devices used for xMD; the hand-held laser gun (left) and the broad-spectrum flashlamp (right); B: Representative image of IHC-stained cell procurement using xMD (original magnification 40×). Positively stained normal esophageal epithelial cells labeled by cytokeratin (AE1/AE3) are shown in the left panel. The EVA membrane with the dissected epithelial cells following xMD is shown on the right. In this dissection, the efficiency of xMD was approximately 70%; C: Periostin protein expression analysis by immunoblot illustrating the high specificity of xMD (left). Relative protein expression levels of periostin, presented as the value of the protein normalized to actin (right).

mance of the technology was confirmed, we completed the validation study on two TMAs,

each containing 25 tumor or matched normal cases as described above.

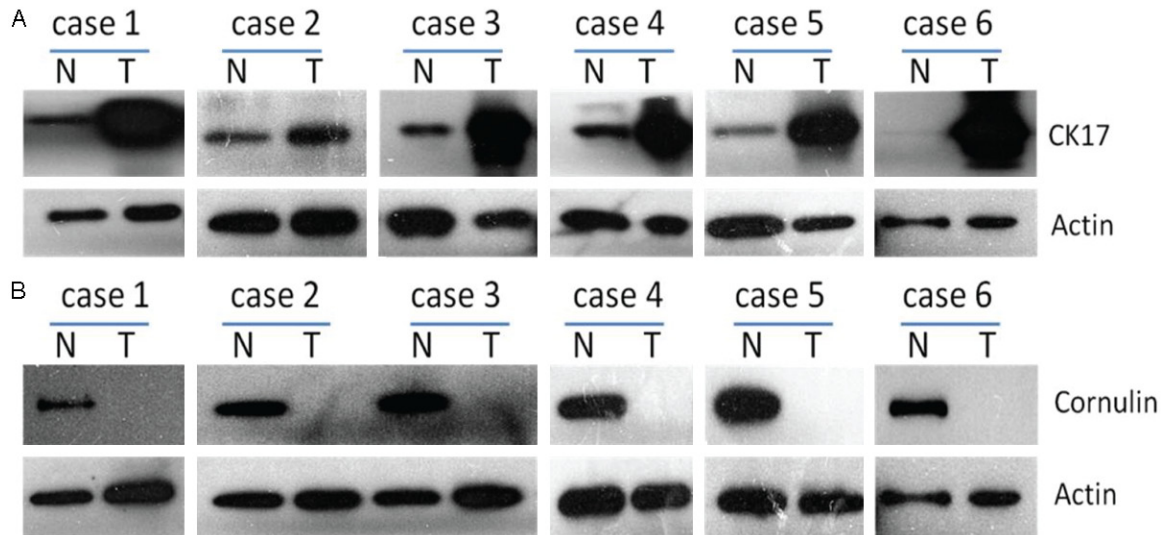


Figure 2. Immunoblot assay showing CK17 (A) and Cornulin (B) expression in normal (N) and tumor (T) cells in six frozen ESCC cases.

Results

Evaluation of technologies

Two xMD devices, a hand-held laser gun and a flashlamp system, were initially evaluated for cell procurement efficiency (**Figure 1**). Microdissection via both light sources was similar, with an average of approximately 50% procurement as assessed via microscopic visualization by a pathologist (Q.D.). However, two factors that affected the efficiency of xMD were identified. One is the staining intensity of the targeted cells and the other is the laser energy applied to the tissue section. Given cells with similar IHC staining intensity, more energy output from the laser source procured more stained cells in less time. And when the energy output was fixed, stronger DAB staining produced greater recovery of target cells.

To establish the specificity of xMD dissections, the expression of periostin, a cell adhesion protein that plays an important role in tumor development and metastasis [9], was measured in both xMD and whole slide scrape samples. In the previous expression array study, periostin mRNA was over-expressed in tumor cells compared to normal esophageal epithelial cells [3]. However, as a secreted protein, periostin was found only in the stroma of esophageal carcinoma but not expressed in tumor cells and normal tissue as determined by IHC staining [3].

We found that periostin expression was weak in the xMD tumor sample and that no protein was detected in either the whole-slide or xMD normal tissue samples. In contrast, over-expression in the whole-slide tumor sample, which included the IHC-positive stroma, showed a relative protein expression (periostin/actin) more than six fold that of the xMD sample (**Figure 1**). This result is consistent with our previous IHC data and demonstrates that the xMD method is highly specific for targeted cells since few stroma cells and little extracellular matrix were isolated, as confirmed visually and via the periostin analysis.

Immunoblot analysis

Six frozen human cases with matched normal and tumor esophageal tissues were used for protein expression assessment of CK17, cornulin, EpCAM and CD44 by immunoblot assay, using actin as a normalizer. xMD was first utilized to isolate the normal or tumor epithelium from each case and the total protein from the captured cells was isolated. The concentrations of the microdissected samples ranged from 0.22 $\mu\text{g}/\mu\text{l}$ to 0.49 $\mu\text{g}/\mu\text{l}$, with an average of 0.31 $\mu\text{g}/\mu\text{l}$.

When evaluating the protein expression of CK17, we found that, except for case #2, CK17 was highly expressed in cancer cells compared with normal epithelial cells (**Figure 2**, panel A),

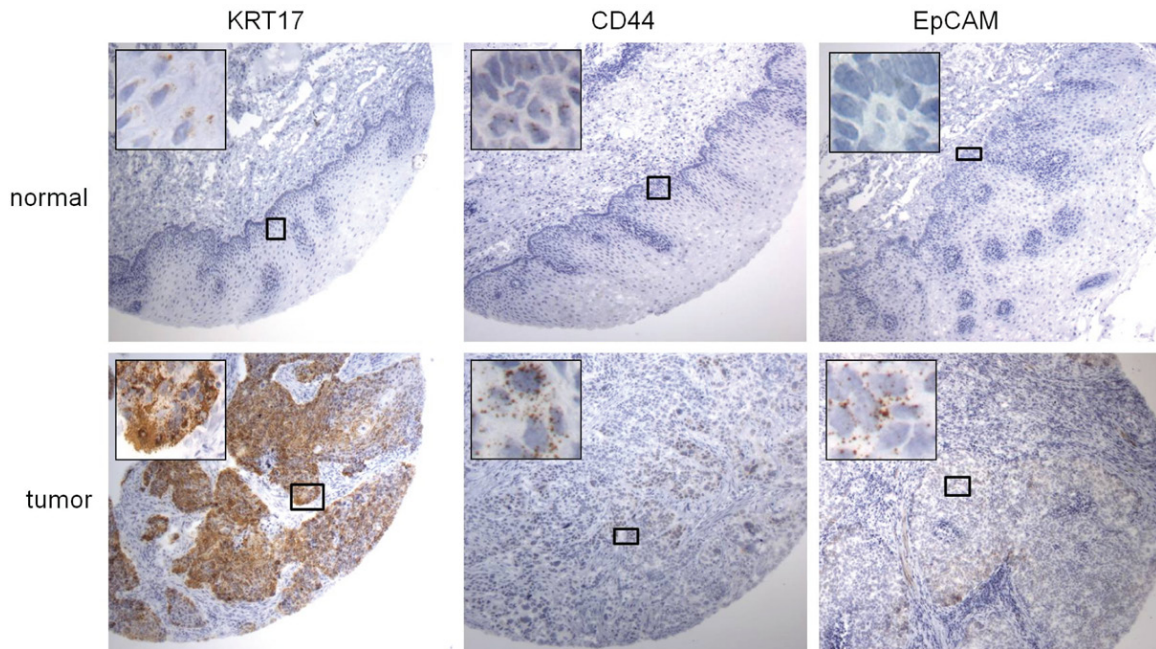


Figure 3. RNA *in situ* hybridization of KRT17, CD44 and EpCAM in a tissue microarray core using the RNAscope 2.0 FFPE Assay kit. KRT17 (left), CD44 (middle), and EpCAM (right). The upper panel shows representative expression in normal epithelium and the lower panel illustrates expression patterns in matched tumor cells from the same case (magnification $\times 100$). The insets show enlarged areas in the highlighted frame (magnification $\times 400$). Positive staining was identified as brown punctuate dots present in the section.

with at least 6-fold over-expression in 5 out of 6 cases. Even in case #2, the CK17 expression in tumor demonstrated 1.5 fold over-expression compared to normal cells. However, in contrast to CK17, cornulin was not observed in any tumor samples but was strongly detected in the normal squamous epithelium (**Figure 2**, panel B). These data indicate that cornulin expression decreases during the development of ESCC and validate the mRNA expression pattern observed in the previous microarray study [3]. Both EpCAM and CD44 protein expression was not detected via the immunoblot assay (data not shown).

RNAscope assay for mRNA expression

RNA *in situ* hybridization, via RNAscope, was utilized to validate the gene expression of KRT17, CD44, and EpCAM within the complex two-dimensional architecture of the tissue. Unfortunately, it was not possible to evaluate CRNN (cornulin) expression via this assay due to the lack of commercially available target probes. Thus, in order to assess the sensitivity of the RNAscope technology, we initially carried out a pilot study with three FFPE ESCC cases,

evaluating KRT17, CD44 and EpCAM expression. In agreement with the western-blot analysis, KRT17 was strongly positive in tumor cells, yet barely visible in both tumor stroma and the matched normal region (3/3). CD44 demonstrated a low expression level in the tumor (3/3) and adjacent normal basal cells (2/3). EpCAM was weakly to moderately stained in tumor cells, while showing little to no expression in the matched normal regions (3/3).

Next, an FFPE esophageal TMA was evaluated by the RNAscope technology for KRT17, CD44, and EpCAM. This analysis further confirmed the initial observations with the pilot study. The RNAscope result for KRT17 demonstrated strong positive staining in the cytoplasm of tumor cells (**Figure 3**). This result is consistent with the elevated protein level observed in the immunoblot results presented above. Weak EpCAM and CD44 expression was observed in the cytoplasm of tumor cells, while in normal epithelium, no EpCAM expression was found, and CD44 expression was only sparsely distributed in some basal cells (**Figure 3**). Each core was manually evaluated and scored according to the manufacturer's semi-quantitative scor-

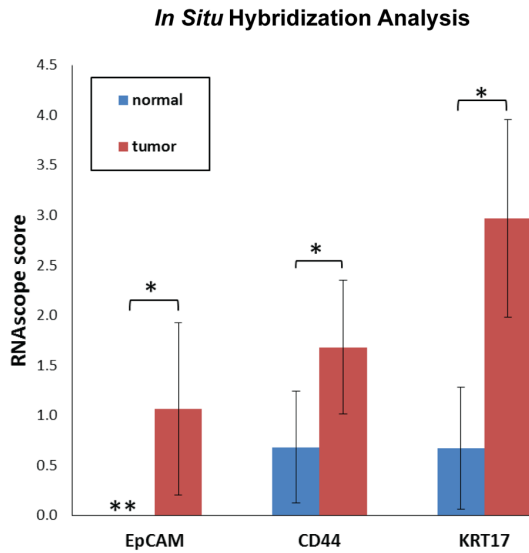


Figure 4. RNA *in situ* hybridization demonstrates differential mRNA expression of EpCAM, CD44, and KRT17 between normal epithelial cells and tumor cells. The gene expression in each TMA core was evaluated and scored according to the manufacturer's instruction. The results of statistical analysis of the difference between matched normal and tumor cells are shown: *, $p < 0.05$; **, no expression was observed.

ing guideline (**Figure 4**). Unfortunately, some cases could not be completely evaluated for each target due to either the lack of epithelium in the core or the exhaustion of tissue for that particular core. However, the paired t-test analysis revealed significant expression differences in matched tumor/normal samples, with a p -value of 2.90×10^{-4} , 1.13×10^{-5} and 4.35×10^{-9} for EpCAM, CD44 and KRT17, respectively.

Discussion

The present study demonstrates validation of several new potential biomarkers for ESCC including; KRT17, cornulin, CD44 and EpCAM, using immunoblot and *in situ* hybridization technologies. The high-throughput xMD technology was utilized to obtain sufficient amounts of epithelium for immunoblot protein expression analysis of KRT17 and cornulin. However, the western blot platform is not ideal for the observation of low expressing proteins due to the conglomeration of the entire cellular lysate and the abundance/sensitivity requirements of the antibody-antigen interaction. Therefore, for the expression analysis of CD44 and EpCAM, it was necessary to employ a more sensitive

technology, thus *in situ* hybridization via RNAscope was utilized. Investigators should utilize and adapt validation strategies based on the goals of their study, availability or reagents, abundance level of their target transcript or protein and technological capabilities of the methods they employ.

Overall, the efficiency and specificity of xMD was shown to be quite high. However, according to our experiences, appropriate optimization for a given study is still required to acquire enough protein for immunoblot assays. Since xMD is an IHC-based microdissection technique, it is important to optimize the staining process to reduce background, and to reduce the time of the staining process in order to limit the loss of protein. Modifications that can improve the efficiency of microdissection include: fixing the frozen section in 70% ethanol for 4 or 5 minutes; limiting the times of PBS washing; and reducing the incubation time of primary and secondary antibodies to half an hour each. Furthermore, xMD was performed on the same day of immunostaining to procure proteins of the highest quality. Decreasing background staining requires optimization of each individual primary antibody concentration and the monitoring of the DAB development step under microscopic visualization. An interesting detail is that the solution derived from protein extraction from the membrane after xMD displayed a weak brown color, presumably from the DAB stain. This may have negatively affected the subsequent BCA assay since even when loading equal amounts of calculated protein we observed a slight variation in the immunoblot housekeeping actin level. To overcome this problem, we optimized the protein loading amount to produce comparable actin normalization bands in subsequent immunoblots.

By performing immunoblot analysis with six frozen cases and RNA *in situ* hybridization on a FFPE TMA, we validated that KRT17 (CK17) is highly expressed in esophageal squamous cell carcinoma (ESCC) cells, but not in normal epithelial cells. In contrast, cornulin expression, assessed by immunoblot, is decreased in cancer cells compared to normal. These results are consistent with the previous cDNA microarray data [3]. KRT17 (CK17) has also been reported in several studies to be correlated with epithelial proliferation and tumor progression [10, 11]; whereas, cornulin is associated with squa-

mous differentiation and only expressed in differentiated epithelium [12]. Our data are consistent with these previous studies [13], and is the first study verifying this phenomenon using microdissected cells and immunoblot analysis, an important technical milestone. As a general matter, combining xMD and immunoblotting allows investigators the ability to assess both the quantitative (densitometry measurement) and qualitative (band size, post-translational modifications) aspects of a protein in a specifically dissected cell population.

We also observed that CD44 and EpCAM transcript levels were more abundant in ESCC than in normal epithelial cells via the RNAscope technology, supporting their role in the development and progression of ESCC, consistent with previously published studies [5, 14, 15]. However, a thorough understanding of the involvement of these genes in esophageal tumorigenesis is not yet clear. Interestingly, EpCAM is highly expressed in tumor-associated stroma in prostate cancer as assessed by both microarray and IHC staining [16, 17]. However, in the current study, we did not observe EpCAM mRNA expression in stromal cells adjacent to ESCC epithelium in either the whole tissue sections or the TMA cores, which may indicate that EpCAM plays a unique role in the tumor micro-environment of prostate cancer, yet a different role in ESCC. From the RNAscope data, CD44 was weakly expressed in normal squamous epithelial cells, and mostly distributed in basal cells. This result is consistent with the previous cDNA microarray data in which CD44 demonstrated more than a 4-fold higher expression in normal basal cells than in differentiated cells [3], implicating its role as a putative stem cell marker. Interestingly, CD44 showed a statistically significant increase in expression in tumor cells in this study, while this change was not observed in the original microarray study, demonstrating that the two methods each have particular strengths and weaknesses when it comes to more subtle measurements.

Overall, the results from the immunoblot and RNA *in situ* hybridization experiments validated our previous expression microarray data [3]. In addition, xMD was shown to be an effective tool for the rapid procurement of specific cell populations from tissue sections for routine immunoblot analyses to be performed and the RNAscope technology enabled the expression

analysis of low abundant targets. Finally, the immunoblot and RNAscope data provide evidence for KRT17 (CK17), cornulin, CD44 and EpCAM as potential molecular targets for ESCC diagnosis and therapy.

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References

- [1] Kamangar F, Dores GM, Anderson WF. Patterns of cancer incidence, mortality, and prevalence across five continents: defining priorities to reduce cancer disparities in different geographic regions of the world. *J Clin Oncol* 2006; 24: 2137-2150.
- [2] Jemal A, Siegel R, Xu J, Ward E. Cancer Statistics, 2010. *Ca Cancer J Clin* 2010; 60: 277-300.
- [3] Yan W, Shih JH, Rodriguez-Canales J, Tangrea MA, Ylaya K, Hipp J, Player A, Hu N, Goldstein AM, Taylor PR, Emmert-Buck MR, Erickson HS. Identification of unique expression signatures and therapeutic targets in esophageal squamous cell carcinoma. *BMC Res Notes* 2012; 5: 73.
- [4] Went P, Dirnhofer S, Schopf D, Moch H, Spizzo G. Expression and prognostic significance of EpCAM. *J Cancer Molecules* 2008; 3: 169-174.
- [5] Zhao JS, Li WJ, Ge D, Zhang PJ, Li JJ, Lu CL, Ji XD, Guan DX, Gao H, Xu LY, Li EM, Soukiasian H, Koeffler HP, Wang XF, Xie D. Tumor initiating cells in esophageal squamous cell carcinomas express high levels of CD44. *PLoS One* 2011; 6: e21419.
- [6] Tangrea MA, Chuaqui RF, Gillespie JW, Ahram M, Gannot G, Wallis BS, Best CJ, Linehan WM, Liotta LA, Phoida TJ, Bonner RF, Emmert-Buck MR. Expression microdissection: operator-independent retrieval of cells for molecular profiling. *Diagn Mol Pathol* 2004; 13: 207-12.
- [7] Hanson JC, Tangrea MA, Kim S, Armani MD, Pohida TJ, Bonner RF, Rodriguez-Canales J,

- Emmert-Buck MR. Expression microdissection adapted to commercial laser dissection instruments. *Nat Protoc* 2011 Apr; 6: 457-67.
- [8] Shou JZ, Hu N, Takikita M, Roth MJ, Johnson LL, Giffen C, Wang QH, Wang C, Wang Y, Su H, Kong LH, Emmert-Buck MR, Goldstein AM, Hewitt SM, Taylor PR. Overexpression of CD-C25B and LAMC2 mRNA and protein in esophageal squamous cell carcinomas and premalignant lesions in subjects from a high-risk population in China. *Cancer Epidemiol Biomarkers Prev* 2008; 17: 1424-35.
- [9] Morra L, Moch H. Periostin expression and epithelial-mesenchymal transition in cancer: a review and an update. *Virchows Arch* 2011; 459: 465-75.
- [10] Depianto D, Kerns ML, Dlugosz AA, Coulombe PA. Keratin 17 promotes epithelial proliferation and tumor growth by polarizing the immune response in skin. *Nat Genet* 2010; 42: 910-4.
- [11] Ide M, Kato T, Ogata K, Mochiki E, Kuwano H, Oyama T. Keratin 17 expression correlates with tumor progression and poor prognosis in gastric adenocarcinoma. *Ann Surg Oncol* 2012; 19: 3506-14.
- [12] Contzler R, Favre B, Huber M, Hohl D. Cornulin, a new member of the "fused gene" family, is expressed during epidermal differentiation. *J Invest Dermatol* 2005; 124: 990-7.
- [13] Pawar H, Maharudraiah J, Kashyap MK, Sharma J, Srikanth SM, Choudhary R, Chavan S, Sathe G, Manju HC, Kumar KV, Vijayakumar M, Sirdeshmukh R, Harsha HC, Prasad TS, Pandey A, Kumar RV. Downregulation of cornulin in esophageal squamous cell carcinoma. *Acta Histochem* 2013; 115: 89-99.
- [14] Roye GD, Myers RB, Brown D, Poczatek R, Beenken SW, Grizzle WE. CD44 expression in dysplastic epithelium and squamous-cell carcinoma of the esophagus. *Int J Cancer* 1996; 69: 254-8.
- [15] Stoecklein NH, Siegmund A, Scheunemann P, Luebke AM, Erbersdobler A, Verde PE, Eisenberger CF, Peiper M, Rehders A, Esch JS, Knoefel WT, Hosch SB. Ep-CAM expression in squamous cell carcinoma of the esophagus: a potential therapeutic target and prognostic marker. *BMC Cancer* 2006; 6: 165.
- [16] Richardson AM, Woodson K, Wang Y, Rodriguez-Canales J, Erickson HS, Tangrea MA, Novakovic K, Gonzalez S, Velasco A, Kawasaki ES, Emmert-Buck MR, Chuaqui RF, Player A. Global expression analysis of prostate cancer-associated stroma and epithelia. *Diagn Mol Pathol* 2007; 16: 189-97.
- [17] Mukherjee S, Richardson AM, Rodriguez-Canales J, Ylaya K, Erickson HS, Player A, Kawasaki ES, Pinto PA, Choyke PL, Merino MJ, Albert PS, Chuaqui RF, Emmert-Buck MR. Identification of EpCAM as a molecular target of prostate cancer stroma. *Am J Pathol* 2009; 175: 2277-87.