ORIGINAL RESEARCH

Desirée Hao, MD^{1,2}
Tien Phan, MD^{2,4}
Amanda Jagdis, MD³
Jodi E. Siever, MSc⁵
Alexander C. Klimowicz, PhD⁶
Janessa J. Laskin, MD^{3,7}
Thomas A. Thomson, MD⁸
M. Sarah Rose, PhD⁹
Stephanie K. Petrillo, MSc⁶
Anthony M. Magliocco, MD¹⁰
Harold Y. Lau, MD^{2,4}

¹ Department of Medical Oncology, Tom Baker Cancer Centre, Calgary, Alberta, Canada

² Faculty of Medicine, University of Calgary, Calgary, Alberta, Canada

³ Department of Internal Medicine, Faculty of Medicine, University of British Columbia, Vancouver, British Columbia, Canada

⁴ Department of Radiation Oncology, Tom Baker Cancer Centre, Calgary, Alberta, Canada

⁵ Senior Analyst/Biostatistics, Research & Innovation, Population, Public, and Aboriginal Health, Alberta Health Services

⁶ Department of Pathology, Tom Baker Cancer Centre, Calgary, Alberta, Canada

⁷ Department of Medical Oncology, British Columbia Cancer Agency – Vancouver

⁸ Pathologist, British Columbia Cancer Agency – Vancouver and Faculty of Medicine, University of British Columbia

⁹ Rho - Sigma Scientific Consultants Calgary, Calgary, AB, Canada

¹⁰ Department of Anatomic Pathology, H. Lee Moffitt Cancer Center, Tampa, Florida, U.S.A.

Manuscript submitted 24th April, 2014

Manuscript accepted 9th September, 2014

Clin Invest Med 2014; 37 (5): E320-E330.

Evaluation of E-cadherin, β -catenin and vimentin protein expression using quantitative immunohistochemistry in nasopharyngeal carcinoma patients

Abstract

Purpose: Aberrant expression of proteins involved in epithelial-to-mesenchymal transition have been described in various cancers. In this retrospective study, we sought to evaluate E-cadherin, β -catenin and vimentin protein expression in non-metastatic nasopharyngeal (NPC) patients treated with curative intent, examine their relationship with each other, and with clinical outcome measures.

Methods: Pre-treatment formalin-fixed paraffin-embedded biopsies of 140 patients treated between January 2000 and December 2007 were assembled into a tissue microarray (TMA). Automated quantitative immunohistochemistry (AQUA®) was performed on sequential TMA sections stained with fluorescent-labeled antibodies against E-cadherin, β -catenin and vimentin. Cox proportional hazards regression was used to estimate the effect of cytoplasmic vimentin, cytoplasmic E-cadherin, β -catenin nuclear/cytoplasmic ratio expression on overall survival and disease-free survival.

Results: The average age of the patients was 51.7 years (SD=12.1; range 18-85), 66% were male, 71% had a KPS $\geq$ 90% at the start of treatment and 65% had stage III/IV disease. After adjusting for performance status, WHO and stage, high E-cadherin levels over the 75th percentile were found to produce a significantly increased risk for both a worse overall survival (HR = 2.53, 95% CI 1.21, 5.27) and disease free survival (DFS; HR = 2.14, 95%CI 1.28, 3.59). Vimentin levels over the first quartile produced an increased risk for a worse DFS (HR = 2.21, 95% CI 1.11, 4.38). No association was seen between β -catenin and survival.

Conclusion: In this cohort of NPC patients, higher levels of E-cadherin and higher levels of vimentin were associated with worse outcomes. Further work is needed to understand the role of these epithelial mesenchymal transition proteins in NPC.

Correspondence to:

Desirée Hao
Department of Medical Oncology
Tom Baker Cancer Centre
1331 29th St NW
Calgary, Alberta, Canada, T2N 4N2
E-Mail: Desiree.Hao@albertahealthservices.ab.ca

Epithelial mesenchymal transition (EMT) which underlies embryonic development, increasingly appears to be critical in the process of tumour invasion and metastases [1]. Although EMT is regulated by a complex interplay of signaling pathways, a hallmark of EMT is the down regulation of E-cadherin, mis-localization of β -catenin and nuclear expression of vimentin [2]. E-cadherin is a transmembrane glycoprotein that is involved in mediating cell-cell adhesion between adjacent epithelial cells in various tissues [3, 4]. Loss of E-cadherin has been related to tumour aggressiveness and increased rates of metastasis in breast, gastric, hepatocellular, bladder and prostate cancer [5-10]. β -catenin is a multi-functional protein involved in the cadherin-mediated cell-cell adhesion system [11]. It associates with the cytoplasmic portion of E-cadherin and the actin cytoskeleton via α -catenin to form the adherens junction [12, 13]. β -catenin is also an important effector of Wnt signaling in the nucleus, where it regulates the expression of genes involved in cell growth. Unbound cytoplasmic β -catenin is targeted for proteosomal degradation in the absence of Wnt signaling. E-cadherin is thought to antagonize β -catenin signaling by binding to it, thus keeping the level of cytoplasmic β -catenin low and unavailable for downstream activation of the Wnt pathway [14, 15]. Loss of E-cadherin expression has been associated with increased localization of β -catenin to the nucleus [15, 16]. Vimentin is a type III intermediate filament protein normally found in mesenchymal cells and is expressed in epithelial cells that are migrating during embryogenesis, organogenesis, wound healing and tumour invasion [17]. The vimentin promoter is a target of the β -catenin/T-cell factor pathway, suggesting that the functional regulation of epithelial cells is involved in tumour invasion and/or metastasis [18].

Since nasopharyngeal cancers (NPC) commonly invade surrounding tissue or metastasize to cervical lymph nodes early in the natural history of the disease, we sought to characterize the protein expression of E-cadherin, β -catenin and vimentin, three proteins involved in EMT, in non-metastatic NPC patients treated with curative intent. We explored the relationship among E-cadherin, β -catenin and vimentin, and their relationship with clinical outcome measures.

Materials and Methods

Study Population, TMA Construction and Analysis

This study was approved by the University of Calgary Conjoint Health Research Ethics Board and the University of British Columbia, British Columbia Cancer Agency (BCCA) Research Ethics Board. Between January 2000 and December 2007, 137 patients with non-metastatic NPC treated with

curative intent radiation $\pm$ platinum chemotherapy at British Columbia Cancer Agency and Tom Baker Cancer Centre were identified with retrievable biopsy samples, either from the primary site or from a nodal metastasis. Tissue microarrays (TMAs) were constructed from duplicate 0.6 mm cores of pre-treatment formalin-fixed paraffin-embedded specimens. Fluorescent immunohistochemistry was performed on sequential TMA sections that were co-stained with antibodies against E-cadherin (1:200, Rabbit clone 24E10, Cell Signaling, Danvers, MA) or β -catenin (1:1000 mouse clone B-cat-1, Dako Cytomation, Glostrup, Denmark) and vimentin (MAB2105 rat; R&D Systems, Minneapolis, MN) and pan-cytokeratin (AE1/AE3 or rabbit anti-cow wide spectrum screening antibody, respectively; Dako Cytomation). Antibody signals was amplified and visualized using a TSA-Plus CY5 (E-cadherin and β -catenin) or Cy3 (vimentin) Tyramide Signal Amplification kit (PerkinElmer, Waltham, MA) and pan-cytokeratin was visualized using a goat anti-primary Alexa 555 conjugated secondary antibody (Invitrogen, Carlsbad, CA). After staining, TMA slides were mounted in ProLong Gold mounting media with diamidino-phenylindole to visualize the nucleus (Invitrogen). TMAs were then scanned using a HistoRx PM-2000 image analysis platform. AQUAnalysis™ software (version 2.3.4.1) was used to create a "mask" to isolate the epithelial/tumour cells within the tissue cores. E-cadherin, β -catenin or vimentin pixel intensity was measured within the tumour area (tAQUA), cytoplasmic tumour area (cAQUA) or the nuclear tumour area (nAQUA).

Statistical Analysis

The nuclear/cytoplasm β -catenin ratio (n/cBC) was obtained by dividing the nAQUA scores by the cAQUA scores [26]. For vimentin and E-cadherin, cAQUA scores were used for analysis (tumour cell cytoplasmic vimentin and tumour cell cytoplasmic E-cadherin, respectively), and both were Z-score standardized between the Calgary and the Vancouver cohorts. All three biomarkers were stratified into quartiles with the highest category considered to have the highest expression of the protein.

Categorical variables were expressed as a frequency and percentage; patient age was expressed as mean $\pm$ standard deviation. The correlation between the biomarkers was assessed using the Spearman rank correlation coefficient. The relationship between each biomarker and other variables (T-stage, N-stage, WHO class, and grade) was assessed using ANOVA after appropriate transformations were applied to achieve an approximately Normal distribution or the Kruskal Wallis non-parametric test when an appropriate transformation could not be found.

TABLE 1. Demographic and treatment characteristics for the overall sample

Characteristic	Included Patients (N = 137)	Excluded Patients (N=142)	P
Age in years (Mean (SD))	52 (12.1)	52 (13.3)	0.453
Male gender	No. (%) 91 (66%)	99 (70%)	0.555
Stage I/II III/IV	48 (35%) 89 (65%)	60 (43%) 81 (57%)	0.819
KPS $\geq$ 90%	97 (71%)	120 (84%)	0.006
WHO type 1 2 3	16 (12%) 38 (29%) 79 (59%)	11 (8%) 30 (22%) 97 (70%)	0.164
RT protocol 3D conformal(66-70 Gy, 33-35 fractions) IMRT(70 Gy, 33-35 fractions)	89 (67%) 43 (33%)	101 (75%) 34 (25%)	0.183
Treatment modality RT only CCRT Adjuvant chemotherapy	80 (58%) 38 (28%) 19 (14%)	97 (68%) 31 (22%) 14 (10%)	0.222
T stage T1 T2 T3 T4	49 (36%) 39 (29%) 21 (15%) 28 (20%)	55 (39%) 43 (30%) 16 (11%) 28 (20%)	0.709
N stage N0 N1 N2/3	44 (32%) 36 (26%) 57 (42%)	45 (32%) 33 (23%) 64 (45%)	0.864
2 year locoregional control rate	113 (82%)	126 (89%)	0.136
Total Death NPC-related	36 (26%) 35/36 (97%)	34 (24%) 32/24 (94%)	0.811

Note: Denominator varies slightly due to missing data.

Overall survival (OS) was defined as the date of diagnosis to the date of death or last follow-up visit, with patients censored at their last follow-up visit. Disease-free survival (DFS) was defined as the date of diagnosis to the date of relapse, progression, death, or last follow-up visit and similarly censored at last follow-up visit. The Kaplan-Meier method was used to estimate 5 year OS and DFS for the sample overall and by subgroups based on the quartiles of each biomarker.

Cox proportional hazards regression was used to estimate the effect of cytoplasmic vimentin, cytoplasmic E-cadherin, β -catenin nuclear/cytoplasmic ratio expression on both OS and DFS. The assumption of linearity for each biomarker in continuous form was assessed using martingale-based residuals; the proportional hazards assumption was assessed using Cox-Snell residuals. Since there was clear evidence against the assumption of linearity for all three biomarker variables, they were entered into the model as categorical variables. Unadjusted hazard ra-

tios were first calculated using a univariable Cox regression analysis. A multivariable Cox regression model was then developed to estimate hazard ratios for each biomarker after controlling for potential confounding variables: stage (1/2, 3/4), Karnofsky Performance Scale (KPS 60/70/80, KPS 90/100) and World Health Organization (WHO) type (WHO 1, WHO 2/3). Due to some small/empty cells, a combined variable KPS/WHO was created with four categories: (i) (KPS 60/70/80 and WHO1; (ii) KPS 90/100 and WHO 1; (iii) KPS 60/70/80 and WHO 2/3; and, (iv) KPS 90/100 and WHO2/3). When a high correlation between two or more variables (e.g., treatment and stage) was observed, the variable with the most explanatory power was included in our model. Potential confounding variables were assessed first by examining the effect of each variable on survival, based on the Likelihood Ratio Statistic, and then by examining the effect on the HR for each biomarker by fitting a regression model with the biomarker and the potential confounding variable. Variables significant at $p < 0.2$ and variables that displayed confounding were included in the full regression model. Subsequently, variables were removed if they were not significant at $p < 0.05$ and were not confounding variables. For simplicity, in the multivariable model, adjacent groups within biomarker categories with similar hazard ratios (and 95% CI) and survival functions were collapsed. All analyses were based on available data and conducted in Stata SE, Version 12 (StataCorp LP, College Station, TX).

Results

Patient characteristics

Two hundred seventy nine non-metastatic NPC patients were treated at the BC Cancer Agency ($n=225$) or Tom Baker Cancer Centre ($n=54$) between January 2000 and December 2007. One hundred thirty seven patients (49%) were included with the remainder excluded due to lack of adequate tissue. Table 1 summarizes the clinical and pathologic features, treatment and recurrence details of the study population. The mean age of the study population was 51.7 years ($SD=12.1$; range 18-85), 91/137 (66%) of the patients were male, and 97/137 (71%) of the patients had a KPS $\geq 90\%$ at the start of treatment. WHO type 3 NPC was the most common histologic subtype (WHO type 1/2/3=12%, 29%, 59%, respectively). The majority (89/137=65%) of patients had stage III/IV disease. Fifty seven of 137 (42%) patients received platinum-based CRT; the remainder were treated with radiation alone. With a median follow-up of 48 months (range 3 to 120), the 5 year overall survival (OS) was 70%. In terms of the baseline and demographic

characteristics, the included versus excluded patients were similar with respect to age at diagnosis, cancer center, T-stage and N-stage, but there were more KPS ≥ 90 patients among those excluded (84% compared with 70%, $p = 0.006$). Since there was no evidence of a difference in the 2 year locoregional control rate and NPC deaths, we can assume that the included patients were a representative sample of the total patient population.

E-cadherin, β -catenin and vimentin expression

Figure 1 shows examples of E-cadherin, β -catenin and vimentin staining using the HistoRx AQUA™ technology platform. The distributions of the biomarkers within each quartile are described in Table 2. The distribution of the z scores for vimentin and E-cadherin illustrates that there is sufficient variability between the categories of protein expression and hence the ability of these two biomarkers to differentiate in terms of survival. There was less variability of β -catenin in this group of patients,

TABLE 2. Distribution of the biomarkers within and across quartiles

	Group	Quartiles	N	Mean	Min	Max	Range
Vimentin	1	< Q1	31	-0.89	-1.28	-0.66	0.62
	2	Q1-Q2	31	-0.51	-0.65	-0.33	0.32
	3	Q2-Q3	31	0.01	-0.32	0.34	0.66
	4	> Q3	32	1.35	0.36	4.6	4.24
	Total		125	0.00	-1.28	4.6	5.88
E-cadherin	1	< Q1	32	-1.30	-2.64	-0.57	2.07
	2	Q1-Q2	33	-0.24	-0.55	0.04	0.59
	3	Q2-Q3	32	0.30	0.05	0.63	0.58
	4	> Q3	33	1.21	0.66	2.45	1.79
	Total		130	0.00	-2.64	2.45	5.09
Beta-catenin	1	< Q1	34	0.53	0.47	0.55	0.08
	2	Q1-Q2	34	0.58	0.55	0.59	0.04
	3	Q2-Q3	34	0.60	0.59	0.62	0.03
	4	> Q3	35	0.65	0.62	0.72	0.10
	Total		137	0.59	0.47	0.72	0.25

Q1, Q2, Q3 = Quartile 1, Quartile 2, Quartile 3

FIGURE 1. Examples of ERCC1 (FL297) staining using the HistoRx AQUA™ technology platform. The upper panels immunofluorescent staining and quantitative analysis of E-cadherin (24E10) and β -catenin (B-cat-1) protein expression in NPC patient samples using the HistoRx platform. Left: Antibody controls, with and without primary antibodies targeting E-cadherin and β -catenin on normal human colon. Right: Representative examples of normal nasopharyngeal epithelium in the top panels, a low expressing NPC in the mid panels, and a high expressing NPC in the bottom panels. DAPI (blue) = nuclei, pan-cytokeratin (green) = epithelial/tumour cells, and E-cadherin/ β -catenin (red).

TABLE 3. Univariable Cox regression analysis of OS and DFS by biomarkers

Biomarkers	OS			DFS		
	Hazard Ratio	95% CI	LRS p	Hazard Ratio	95% CI	LRS p
E-cadherin Cytoplasmic						
<Q1	ref	ref		ref	ref	
Q1 – Q2	0.68	0.22-2.09		1.12	0.52-2.41	
Q2 – Q3	0.97	0.35-2.68	0.079	1.36	0.65-2.86	0.050
$\geq$ Q3	2.21	0.92-5.27		2.38	1.20-4.71	
Vimentin Cytoplasmic						
<Q1	ref	ref		ref	ref	
Q1 – Q2	2.65	0.92-7.63	0.309	2.36	1.13-4.92	0.089
Q2 – Q3	1.89	0.62-5.77		1.40	0.63-3.13	
$\geq$ Q3	1.85	0.60-5.66		2.00	0.94-4.24	
Beta-catenin N/C Ratio						
<Q1	ref	ref		ref	ref	
Q1 – Q2	1.06	0.38-2.92	0.693	1.30	0.62-2.76	0.649
Q2 – Q3	1.64	0.63-4.22		1.53	0.74-3.18	
$\geq$ Q3	1.06	0.38-2.92		1.49	0.72-3.09	
Potential Confounding Variables						
Stage						
1/2	ref	ref	0.001	ref	ref	0.011
3/4	3.87	1.51-9.96		1.97	1.14-3.42	
WHO + KPS						
WHO 1, KPS 60/70/80	ref	ref		ref	ref	
WHO 1, KPS 90/100	0.13	0.03-0.64	0.001	0.14	0.03-0.68	0.064
WHO 2/3, KPS 60/70/80	0.25	0.09-0.69		0.38	0.15-0.99	
WHO 2/3, KPS 90/100	0.11	0.04-0.28		0.36	0.15-0.84	

Note: ref=reference group; Q1, Q2, Q3= Quartile 1, Quartile 2, Quartile 3

which is another indicator that it is probably not a good biomarker for these patients.

Correlation between biomarkers and with tumour-related factors

The pairwise correlation among the three biomarkers was low: E-cadherin and β -catenin ($\rho = 0.289$); E-cadherin and vimentin ($\rho = -0.112$); and β -catenin and vimentin ($\rho = 0.052$). There was no apparent relationship between any of the biomarkers with T-stage (E-cadherin $p = 0.204$; vimentin $p = 0.776$; β -catenin $p = 0.208$), N-stage (E-cadherin $p = 0.205$; vimentin $p = 0.517$; β -catenin $p = 0.758$), WHO class (E-cadherin $p = 0.294$; vimentin $p = 0.646$; β -catenin $p = 0.231$), or grade (E-cadherin $p = 0.793$; vimentin $p = 0.244$; β -catenin $p = 0.597$).

Relationship between Biomarkers and Survival

Univariable Cox regression analysis using quartiles for each biomarker indicated a potential relationship between E-cadherin and OS; E-cadherin and DFS; and, vimentin and DFS (Table 3). After collapsing adjacent quartiles with similar hazard ratios for the potential biomarkers, further univariable Cox regression analysis showed a probable relationship between E-cadherin and OS for patients in categories <Q3 *vs.* $\geq$ Q3 (HR = 2.48; 95% CI = 1.25, 4.92; LRS $p = 0.012$), between E-cadherin and DFS for patients in categories <Q3 *vs.* $\geq$ Q3 (HR = 2.07; 95% CI = 1.24, 3.45; LRS $p = 0.008$) and between Vimentin and DFS for patients in categories <Q1 *vs.* $\geq$ Q1 (HR = 1.91; 95% CI = 0.99, 3.66; LRS $p = 0.039$) (Figures 2A and 2B; data not tabled).

The decision to combined KPS and WHO into a single categorical variables with four categories in the multivariable model, (i) (KPS 60/70/80 and WHO1, (ii) KPS 90/100 and

TABLE 4. Multivariable Cox regression analysis of OS and DFS for E-cadherin cytoplasmic and DFS for vimentin cytoplasmic

Biomarkers	OS Model for E-cadherin Cytoplasmic			DFS Model for E-cadherin Cytoplasmic			DFS Model for Vimentin Cytoplasmic		
	Hazard Ratio	95% CI	LRS p	Hazard Ratio	95% CI	LRS p	Hazard Ratio	95% CI	LRS p
OS E-cadherin Cytoplasmic									
≤Q3	ref	ref	0.029			0.022			
>Q3	2.53	1.21-5.27							
DFS E-cadherin Cytoplasmic									
≤Q3				ref	ref	0.022			
>Q3				2.14	1.28-3.59				
DFS Vimentin Cytoplasmic									
≤Q1							ref	ref	0.025
>Q1							2.21	1.11-4.38	
Potential Confounding Variables									
Stage									
1/2	ref	ref	0.029	ref	ref	0.018	ref	ref	0.041
3/4	2.77	1.02-7.51		1.93	1.09-3.41		1.81	1.00-3.26	
WHO + KPS									
WHO 1, KPS 60/70/80	ref	ref	0.008				ref	ref	0.161
WHO 1, KPS 90/100	0.13	0.02-0.72					0.15	0.03-0.82	
WHO 2/3, KPS 60/70/80	0.20	0.07-0.62					0.35	0.11-1.08	
WHO 2/3, KPS 90/100	0.11	0.04-0.33					0.36	0.13-1.01	

Note: ref=reference group; Q1, Q2, Q3 = Quartile 1, Quartile 2, Quartile 3

WHO 1, (iii) KPS 60/70/80 and WHO 2/3, and (iv) KPS 90/100 and WHO2/3), was empirically based. Both of these variables were potential confounders and the data were fairly sparse with many cells that were small or empty; hence, we decided to collapse adjacent cells in KPS into two categories and WHO into two categories. When the effect on survival was examined, there was a significant interaction between these variables in predicting overall survival. This can be seen in Table 3, where the effect of KPS on overall survival depends upon WHO category. For KPS = 90/100, there was very little effect of WHO on survival (HR = 0.13 and HR = 0.11), but when KPS = 60/70/80 there was a large effect (HR = 1 and HR = 0.25). For interpretation purposes this interaction was presented as HRs for each of the four derived groups rather than as the two main effects and the interaction term, which would not have an intuitive interpretation.

Stage was significantly correlated with chemotherapy treatment ($p < 0.001$); that is, among those treated with chemotherapy, 87% were Stage 3/4 therefore we only adjusted for KPS, WHO and stage. After adjusting for KPS, WHO, and stage increasing values of both E-cadherin and vimentin were

associated with poorer survival (Table 4); however, the cut-off values at which statistical significance was reached differed between the two biomarkers. For E-cadherin, values had to be over the 75th percentile before they produced a significantly increased risk for both a worse OS (HR = 2.53, 95% CI 1.21, 5.27) and DFS (HR = 2.14, 95%CI 1.28, 3.59), whereas for vimentin values over the first quartile produced a significantly increased risk for a worse DFS (HR = 2.21, 95% CI 1.11, 4.38). No further increase in risk was observed for values in the third and fourth quartile of vimentin. No association was seen between β -catenin and survival.

Discussion

Elevated values of E-cadherin ($\geq 75^{\text{th}}$ percentile) produced a significantly increased risk for both a worse OS and DFS, while vimentin scores $\geq 25^{\text{th}}$ percentile were associated with a significantly increased risk for a worse DFS. β -catenin was not associated with adverse patient outcomes. Three previous studies have examined E-cadherin, β -catenin $\pm$ vimentin expression, specifically in NPC, with conflicting results. In contrast to our study, Zheng *et al.* reported that decreased

FIGURE 2. Kaplan-Meier curves of (a) OS and (b) DFS associated with biomarkers

membranous staining of E-cadherin and β -catenin were both associated with advanced stages of diseases and there was a trend towards better survival with high expression of E-cadherin or β -catenin [23]. In a second much smaller cohort of 18 NPC patients, down regulation of E-cadherin expression was correlated with lymph node metastases and poor outcome; however, no correlation was observed between β -catenin expression and outcomes [24]. More recently, Luo *et al.* also evaluated E-cadherin, β -catenin and vimentin in biopsies in a comparably-sized group ($n=122$) of NPC patients. As was seen in this study, Luo also reported that the co-expression of high cytoplasmic E-cadherin and high nuclear vimentin, but not β -catenin expression, was correlated with shorter patient survival in multivariate analyses [25].

Differences in methodology may explain the discordance between our results and some of the existing literature on EMT biomarkers in NPC and other tumour types. We elected to use AQUA technology, which allows for biomarker quantification; whereas traditional IHC, employed in the majority of studies

examining EMT-associated biomarkers, is a more qualitative measure of protein expression. The AQUA™ technique has been validated in prior studies with respect to accuracy, interobserver variability and predictive power against traditional pathologist-based scoring for more commonly recognize proteins such as estrogen receptor and HER2 [26]. Moreover, our findings are in keeping with the largest of three other series evaluating EMT in NPC, despite the fact that they did not use quantitative immunofluorescence. Another potential source of discrepancy may be attributable to examination of EMT biomarkers localized to different compartments within the tumour. We elected to examine cytoplasmic E-cadherin, as the mislocalization of E-cadherin has been associated with tumourigenicity. Similarly, other researchers have reported overall β -catenin, whereas we opted to evaluate the nuclear/cytoplasmic ratio to get an idea of activation of β -catenin transcriptional activity. Lastly, differences in tumour sampling may also contribute to different results. Cells undergoing EMT, commonly reported to occur at the invasive front of many ma-

lignancies [27], may constitute only a small proportion of the total population of tumour cells. Luo *et al.* noted the high expression of cytoplasmic E-cadherin and nuclear vimentin was predominantly localized at the infiltrating tumour edge [24]. In our study, the cores used in the TMA were taken from patient's pre-treatment diagnostic biopsies, which may not necessarily be representative of cells from the invasive front of the tumours. In addition, the cores used to create our TMA came from either the primary tumour site or a lymph node metastasis, depending on availability, which could certainly be another source of heterogeneity. E-cadherin expression has been shown to differ between primary tumour specimens versus lymph node or other distant metastases [22, 38] suggesting that E-cadherin expression may be up- or down-regulated depending on the stage of tumour progression.

Underlying biological differences in the patient populations studied could also contribute to the apparently conflicting literature. Our cohort most closely resembles that reported by Galena *et al.* (n=18) [24] in which there were a greater number of WHO I/II cases, whereas the other two NPC studies were done in Asia where WHO type 3 is predominant [23, 25]. We did not observe a difference in outcome by biomarker status when the analyses was stratified by WHO class (1/2 and 3) making this a less likely factor (data not shown). E-cadherin is typically thought to play a role in the maintenance of cell-cell adhesion and as a tumour suppressor [7, 19, 20]. Usually diminished expression of E-cadherin, reflecting a loss of cell-cell adhesion, is associated with poor outcome; however, there are some studies that have not shown any independent prognostic value for E-cadherin [33, 34] over other known prognostic factors, while other studies have demonstrated that overexpression of the E-cadherin- β -catenin complex is associated with more aggressive phenotypes and a poorer outcome [21, 22, 35, 37]. It may be that the expression of E-cadherin during cancer progression is dynamic and highly contextual [22, 28]. There is emerging evidence that collective cell migration is an important mechanism in cancer invasion [29]. In this type of tumorigenesis, cells move as sheets, strands or clusters rather than individually. Cadherin-based junctions between cells remain present to maintain 'supracellular' properties. In a study looking at oral squamous cell carcinoma cell lines, the cells proliferated well when they were cultured as multicellular aggregates; however, they entered into an apoptotic pathway when suspended as single cells. This observation implies a role for cadherin-mediated intercellular adhesions in the promotion of anchorage-independent growth and suppression of apoptosis [30]. Lastly, there are also other signal transduction pathways that are important in regulating the adhesion and motility of

single tumour cells, and the regulation of collective cell migration, which may be of greater prognostic significance than E-cadherin, β -catenin or vimentin. For instance, Johnson *et al.* recently reported that total silencing of CD151 de-stabilizes E-cadherin-dependent carcinoma cell-cell junctions, and enhances the collective migration of intact tumour cell sheets. This effect was dependent on neither reduced E-cadherin expression nor disruptions in the E-cadherin regulatory complex [31].

Our study was limited by its retrospective design, small sample size and potential selection biases due to tissue availability. In an attempt to address these limitations, we did examine the clinical or pathologic characteristics of the included versus excluded patients. The two groups did not differ with respect to age at diagnosis, cancer center, T-stage, N-stage or overall survival. Although there were more KPS $\geq$ 90 patients among those excluded, the 2 year locoregional control rate and NPC deaths were no different between the included versus excluded patients groups; therefore, we assumed the included patients were a reasonably representative sample of the total patient population. The retrospective design and relatively small sample size also creates inherent limitations in our statistical analyses. For instance, the decision to combine KPS and WHO in the analyses was empirically-based, because both of these variables were potential confounders and the data were fairly sparse. Similarly, we did not adjust for the use of chemotherapy in our analyses because there was a high correlation between the variables treatment and stage; instead, we chose to include the variable with the most explanatory power (namely stage). Lastly, our analyses included several comparisons for each biomarker: initially considering each quartile independently (due to non-linearity), then collapsing adjacent quartiles with similar hazard ratios from initial regression analyses. To account for multiple comparisons, within the limitations of our data, we selected the Likelihood Ratio Statistic (LRS), which provides a single p-value per variable rather than the Wald statistic, which would provide three. Our study is exploratory and, hence, the findings are not conclusive but generate hypotheses worth further exploration.

In summary, we observed that the expression of two proteins involved in EMT, E-cadherin and vimentin, may be associated with outcome. After adjusting for performance status, WHO and stage, tumours with E-cadherin levels over the 75th percentile produced a significantly increased risk for both a worse OS and DFS while tumours with vimentin levels over the first quartile produced an increased risk for a worse DFS. No association was seen between β -catenin and survival. To better understand discordant findings of EMT biomarkers in

NPC and across tumour types, studies with larger sample sizes are needed. AQUA™ technology could be compared with standard immunohistochemical assessment to help clarify optimal methodology for assessing protein expression.

Acknowledgments

This project was funded by a Bridge & Pilot Grant from the Alberta Cancer Foundation.

Special thanks to Drs. Cheryl Ho, Beverly Carter and Ms. Mie Kono for their support /assistance with this project.

References

- Meng F, Wu G. The rejuvenated scenario of epithelial-mesenchymal transition (EMT) and cancer metastasis. *Cancer Metastasis Rev* 2012 DOI 10.1007/s10555-012-9379-3.
- Thiery JP, Sleeman JP. Complex networks orchestrate epithelial-mesenchymal transitions. *Nat Rev Mol Cell Biol* 2006;7:131-142.
- Birchmeier W, Weidner KM, Hulsken J, Behrens J. Molecular mechanisms leading to cell junction (cadherin) deficiency in invasive carcinomas. *Semin.Cancer Biol.* 1993 Aug;4(4):231-239.
- Gumbiner BM. Regulation of cadherin adhesive activity. *J. Cell Biol.* 2000 Feb 7;148(3):399-404.
- Bringuier PP, Umbas R, Schaafsma HE, Karthaus HF, Debruyne FM, Schalken JA. Decreased E-cadherin immunoreactivity correlates with poor survival in patients with bladder tumors. *Cancer Res.* 1993 Jul 15;53(14):3241-3245.
- Umbas R, Isaacs WB, Bringuier PP, Schaafsma HE, Karthaus HF, Oosterhof GO, et al. Decreased E-cadherin expression is associated with poor prognosis in patients with prostate cancer. *Cancer Res.* 1994 Jul 15;54(14):3929-3933.
- Gamallo C, Palacios J, Suarez A, Pizarro A, Navarro P, Quintanilla M, et al. Correlation of E-cadherin expression with differentiation grade and histological type in breast carcinoma. *Am. J. Pathol.* 1993 Apr;142(4):987-993.
- Kanai Y, Oda T, Tsuda H, Ochiai A, Hirohashi S. Point mutation of the E-cadherin gene in invasive lobular carcinoma of the breast. *Jpn. J. Cancer Res.* 1994 Oct;85(10):1035-1039.
- Siitonen SM, Kononen JT, Helin HJ, Rantala IS, Holli KA, Isola JJ. Reduced E-cadherin expression is associated with invasiveness and unfavorable prognosis in breast cancer. *Am. J. Clin. Pathol.* 1996 Apr;105(4):394-402.
- Tamura G, Yin J, Wang S, Fleisher AS, Zou T, Abraham JM, et al. E-Cadherin gene promoter hypermethylation in primary human gastric carcinomas. *J. Natl. Cancer Inst.* 2000 Apr 5;92(7):569-573.
- Miller JR, Moon RT. Signal transduction through beta-catenin and specification of cell fate during embryogenesis. *Genes Dev.* 1996 Oct 15;10(20):2527-2539.
- Kemler R. From cadherins to catenins: cytoplasmic protein interactions and regulation of cell adhesion. *Trends Genet.* 1993 Sep;9(9):317-321.
- Geiger B, Yehuda-Levenberg S, Bershadsky AD. Molecular interactions in the submembrane plaque of cell-cell and cell-matrix adhesions. *Acta Anat. (Basel)* 1995;154(1):46-62.
- Shtutman M, Zhurinsky J, Simcha I, Albanese C, D'Amico M, Pestell R, et al. The cyclin D1 gene is a target of the beta-catenin/LEF-1 pathway. *Proc. Natl. Acad. Sci.U.S.A.* 1999 May 11;96(10):5522-5527.
- Gottardi CJ, Wong E, Gumbiner BM. E-cadherin suppresses cellular transformation by inhibiting beta-catenin signaling in an adhesion-independent manner. *J. Cell Biol.* 2001 May 28;153(5):1049-1060.
- Onder TT, Gupta PB, Mani SA, Yang J, Lander ES, Weinberg RA. Loss of E-cadherin promotes metastasis via multiple downstream transcriptional pathways. *Cancer Res.* 2008 May 15;68(10):3645-3654.
- Satelli A, Li S. Vimentin in cancer and its potential as a molecular target for cancer therapy. *Cell Mol Life Sci* 2001;68:3033-3046.
- Gilles C, Polette M, Mestdagt M, et al. Transactivation of vimentin by beta-catenin in human breast cancer cells. *Cancer Res.* 2003; 63:2658-64.
- Frixen UH, Behrens J, Sachs M, Eberle G, Voss B, Warda A, et al. E-cadherin-mediated cell-cell adhesion prevents invasiveness of human carcinoma cells. *J. Cell Biol.* 1991 Apr;113(1):173-185.
- Pierceall WE, Woodard AS, Morrow JS, Rimm D, Fearon ER. Frequent alterations in E-cadherin and alpha- and beta-catenin expression in human breast cancer cell lines. *Oncogene* 1995 Oct 5;11(7):1319-1326.
- Tomlinson JS, Alpaugh ML, Barsky SH. An intact overexpressed E-cadherin/alpha,beta-catenin axis characterizes the lymphovascular emboli of inflammatory breast carcinoma. *Cancer Res.* 2001 Jul 1;61(13):5231-5241.
- Kowalski PJ, Rubin MA, Kleer CG. E-cadherin expression in primary carcinomas of the breast and its distant metastases. *Breast Cancer Res.* 2003;5(6):R217-22.
- Zheng Z, Pan J, Chu B, Wong YC, Cheung AL, Tsao SW. Downregulation and abnormal expression of E-cadherin and beta-catenin in nasopharyngeal carcinoma: close association with advanced disease stage and lymph node metastasis. *Hum. Pathol.* 1999 Apr;30(4):458-466.
- Galera-Ruiz H, Rios MJ, Gonzalez-Campora R, de Miguel M, Carmona MI, Moreno AM, et al. The cadherin-catenin complex in nasopharyngeal carcinoma. *Eur. Arch. Otorhinolaryngol.* 2011 Sep;268(9):1335-1341.

25. Luo W, Fang W, L S, Yao K. Aberrant expression of nuclear vimentin and related epithelial-mesenchymal transition markers in nasopharyngeal carcinoma. *Int J Cancer* 2012;131:1863-1873.
26. Camp RL, Chung GG, Rimm DL (2002) Automated subcellular localization and quantification of protein expression in tissue microarrays. *Nat Med* 8:1323-1327
27. Bryne M, Boysen M, Alfsen CG et al. The invasive front of carcinomas. The most important area for tumor prognosis? *Anticancer Res* 1998;18:4757-4764.
28. Lau MT, Klausen C, Leung PC. E-cadherin inhibits tumor cell growth by suppressing PI3K/Akt signaling via beta-catenin-Egr1-mediated PTEN expression. *Oncogene* 2011 Jun 16;30(24):2753-2766.
29. Friedl P, Gilmour D. Collective cell migration in morphogenesis, regeneration and cancer. *Nat. Rev. Mol. Cell Biol.* 2009 Jul;10(7):445-457.
30. Kantak SS, Kramer RH. E-cadherin regulates anchorage-independent growth and survival in oral squamous cell carcinoma cells. *J. Biol. Chem.* 1998 Jul 3;273(27):16953-16961.
31. Johnson JL, Winterwood N, DeMali KA, Stipp CS. Tetraspanin CD151 regulates RhoA activation and the dynamic stability of carcinoma cell-cell contacts. *J. Cell. Sci.* 2009 Jul 1;122(Pt 13):2263-2273.
32. Hao D, Lau HY, Eliasziw M, Box A, Diaz R, Klimowicz AC, Shin B, Lees-Miller SP, Magliocco AM. Comparing ERCC1 protein expression, mRNA levels and genotype in squamous cell carcinomas of the head and neck treated with concurrent chemoradiation stratified by HPV status. *Head & Neck* 2012; 34(6):785-91.
33. Lipponen P, Saarelainen E, Ji H, Aaltomaa S, Syrjanen K. Expression of E-cadherin as related to other prognostic factors and survival in breast cancer. *J Pathol* 1994;174:101-109
34. Gonzalez MA, Pinder SE, Wencyk PM, Bell JA, Elston CW, Nicholson RI et al. An immunohistochemical examination of the expression of E-cadherin, alpha- and beta/gamma-catenins and alpha2- and beta1-integrins in invasive breast cancer. *J Pathol* 1999;187:523-529.
35. Zhang Y, Du J, Tian X, Zhong Y, Fang W. Expression of E-cadherin, beta-catenin, cathepsin D, gelatinases and their inhibitors in invasive ductal breast carcinomas. *Chin Med J (Engl)* 2007;120(18):1597-1605.
36. Gillett CE, Miles, DW, Ryder K, Skilton D, Liebman RD, Springall RJ, et al. Retention of the expression of E-cadherin and catenin is associated with shorter survival in grade III ductal carcinoma of the breast. *J Pathol* 2001; 193:433-441.
37. Tan DS, Potts HW, Leong, AC, Gillett CE, Skilton D, Harris WH, et al. The biological and prognostic significance of cell polarity and E-cadherin in grade I infiltrating ductal carcinoma of the breast. *J Pathol* 1999;189:20-27
38. Zhi L, Yi R, Lin SX, Liang YJ, Lian HZ. Association of E-cadherin and β -catenin with metastasis in nasopharyngeal carcinoma. *Chin Med J (Engl)* 2004;117(8):1232-1239