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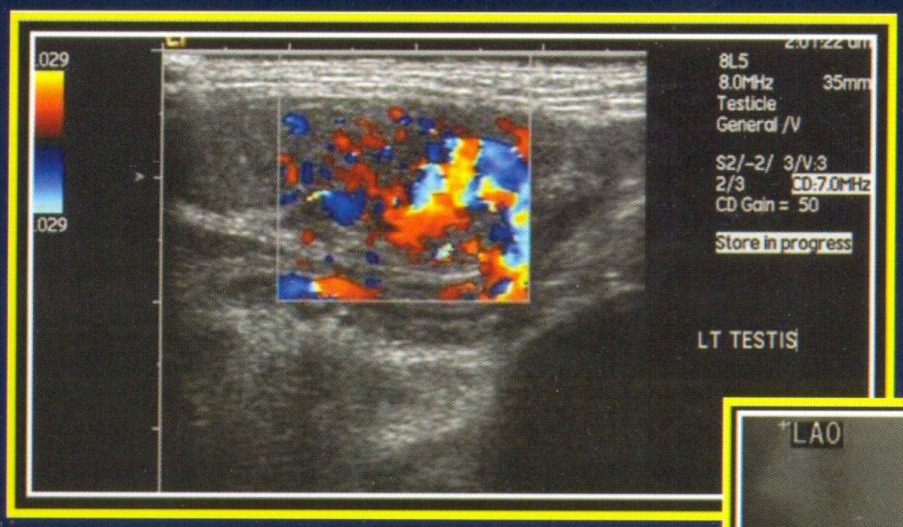

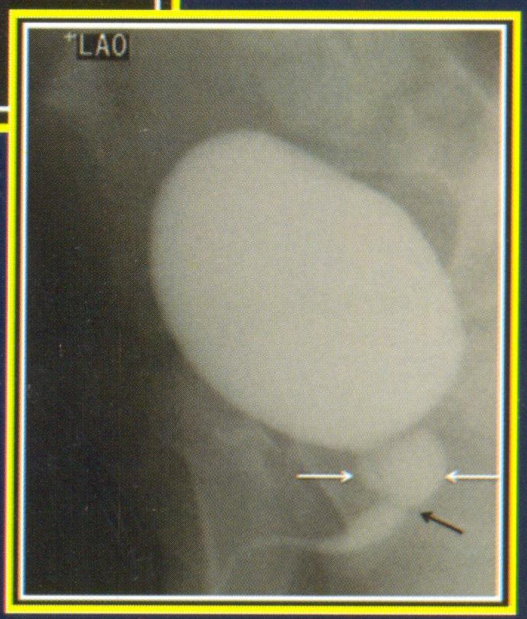

ORIGINAL ARTICLE

Elevated Expression of CD151 Gene in Estrogen Receptor and Progesterone Receptor Positive Breast Carcinoma

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ABSTRAK

CD151 adalah gen tetraspanin yang terlibat dalam perkembangan dan perebakan sel-sel barah. Ekspresi gen ini meningkat pada kanser payudara gred tinggi, reseptor estrogen negatif dan c-erbB-2 positif. Walaubagaimanapun, fungsi biologi dan fenotaip di kalangan pesakit yang mempunyai status barah yang berbeza, status reseptor estrogen (ER), status reseptor progesteron (PR) dan ekspresi c-erbB-2 di Malaysia yang terdiri daripada pelbagai kaum, masih belum dikaji secara teliti. Kami menggunakan teknik *real-time polymerase chain reaction* (qRT-PCR) kuantitatif untuk mengukur ekspresi gen CD151 bagi 45 kanser payudara. Hasil kajian menunjukkan bahawa ekspresi CD151 adalah tinggi pada kanser payudara ER positif (95% interval konfiden) dan PR positif (99% interval konfiden). Sebaliknya, tiada korelasi didapati antara ekpresi CD151 dengan gred tumor atau status c-erbB-2 pada 95% interval konfiden. Hasil kajian setakat ini mencadangkan bahawa CD151 berpotensi sebagai penanda prognostik dan sasaran terapeutik untuk rawatan pesakit payudara estrogen dependen.

Kata kunci: qRT-PCR, ekspresi, kanser payu dara, CD151

ABSTRACT

The tetraspanin gene, CD151 is involved in various tumour cell progression and metastasis. Its expression is increased in high grade, estrogen receptor negative and *c-erbB-2* positive breast cancer. However, the biological function and expression phenotype among different tumour status, estrogen receptor (ER) status, progesterone receptor (PR) status and *c-erbB-2* expression in multi-ethnic Malaysian breast cancer patients has not been well investigated. We used quantitative real-time reverse

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transcriptase polymerase chain reaction (qRT-PCR) to measure the CD151 gene expression in 45 breast cancers. Our preliminary results revealed that CD151 expression is significantly higher in ER positive and PR positive breast cancers at 95% and 99% confidence intervals, respectively. In contrast, there is no significant correlation between CD151 expression and tumour grades or *c-erbB-2* status at 95% confidence interval level. Our preliminary findings suggested that CD151 may be involved in the estrogen responsive pathways. CD151 could be a potential prognostic marker and therapeutic target in the treatment of estrogen dependent breast cancer patients.

Key words: qRT-PCR, expression, breast cancer, CD151

INTRODUCTION

The CD151 gene is a transmembrane molecule that has been characterized as a member of the tetraspanin family. The CD151 transcript consists of 253 amino acids that encode a 28 kDa protein. This gene is mapped to the human chromosome 11p15.1. It is implicated in cell motility, cell adhesion, and stability and formation of hemidesmosomes (Yauch et al. 1998). The role of CD151 gene in cell motility highlighted its function as a promoter in tumour progression and metastasis events (Kohno et al. 2002). Overexpression of CD151 was found to be associated with poor prognosis in non-small cell lung cancer, colon cancer and prostate cancer (Tokuhara et al. 2001; Hashida et al. 2003; Ang et al. 2004). In breast cancer, CD151 is elevated in approximately 31% of human breast cancers and is significantly related to the high-grade (40%) and estrogen receptor negative (45%) subtypes (Yang et al. 2008). The CD151 gene is also a potential marker in predicting histological grading in colon and prostate cancer (Hashida et al. 2003; Ang et al. 2004).

Study on the CD151 mRNA expression in correlation with clinicopathological features of breast cancer is rare. Herein, we investigated the expression of CD151 and correlated its expression with the histological features, ER status, PR status, *c-erbB-2* expression and tumour grades in breast cancer patients.

MATERIALS AND METHODS

Patients and biopsy specimens

A total of 45 breast carcinomas were collected from fresh surgical resections from Kuala Lumpur Hospital, Universiti Kebangsaan Malaysia Medical Centre and Putrajaya Hospital. The biopsies were snap-frozen in liquid nitrogen prior to sample preparation. Tissues were touched on poly-L-Lysine coated slides and subjected for Diff Quick Staining (Imeb Inc.). Microscopic examination of touch preparations verified the presence of at least 70% of cancer cells in all samples. Infiltrating ductal carcinoma (IDC) was diagnosed in 84.4% of the patients (N=39). Other tumours were identified as infiltrating lobular carcinoma (N=1), ductal carcinoma *in-situ* (DCIS) (N=2), mucinous carcinoma (N=1), mixed mucinous & ductal carcinoma (N=1), and IDC with DCIS (N=1). Majority of the cases were from postmenopausal patients. The mean and median ages of the patients are 50.8 and 52.0 years old, respectively. Tumour grade was evaluated using the Bloom-Richardson grading system. Nine, 24 and 12 tumours were

classified as Grade 1, Grade 2 and Grade 3 tumours, respectively. The ER, PR and c-erbB-2 expression status for all samples were determined by immunohistochemistry (IHC) and the results were as evaluated by the reporting pathologists. Tumours were regarded as ER or PR positive if >10% of tumour cells expressed nuclear positivity. On the other hand, strong membrane staining in >10% cells was considered positive for c-erbB-2. Breast carcinomas with known ER, PR and c-erbB-2 expression were used as controls. The clones for ER, PR and c-erbB-2 were 1DS (DAKO), PgR 636 (DAKO) and SP3 (Neomarker), respectively. All IHC procedures were performed according to the manufacturer's recommendation. The clinical and histopathological data for the patients are listed in Table 1.

Ethics approval was obtained from Medical Research & Ethics Committee, Ministry of Health Malaysia to perform this study.

RNA isolation

Minced tissues were placed directly in Trizol Reagent (Invitrogen), homogenized, and total RNAs were isolated and purified through RNeasy columns (QIAGEN) according to the manufacturer's recommendation. The integrity of the purified total RNA was assessed by visualization of the 28S/18S ribosomal RNA ratio on 1% agarose denaturing gel and the quantity was assessed based on absorbance at 260nm and 280nm in Nanodrop.

cDNA synthesis

Total RNA was converted to cDNA using AMV reverse transcriptase [First strand cDNA synthesis kit for RT-PCR (AMV), Roche Diagnostics]. The cDNA synthesis reactions were performed in 20µL volumes containing 200ng of total RNAs, 1X reaction buffer, 5mM $MgCl_2$, 1mM dNTPs, 3.2µg of random primer $p(dN)_6$, 50 units RNase inhibitor and≥ 20 units of AMV reverse transcriptase. Total RNAs with the appropriate volume of nuclease free water were first incubated at 65°C for 15min. The master mix was then added to the tube and incubated at 25°C for 10min, and then at 42°C for 1h. The reaction was then incubated at 99°C for 5min to denature the enzymes. Finally, the reaction was cooled to 4°C for 5min.

Polymerase chain reaction (PCR)

Polymerase chain reaction was performed to amplify GAPDH and CD151 genes. The PCR reaction was carried out in 25µL volumes containing 4-6µg of cDNA generated from breast tumour, 0.4pmole/µL of primers and 1X PCR master mix (Panomics). The reaction mixture was pre-incubated at 95°C for 5 min, followed by 40-45 cycles of amplification at 94°C for 30s, 54°C for 30s and 72°C for 30s. A negative control was included in each PCR to exclude contamination. PCR products for GAPDH and CD151 genes were purified (Qiagen PCR purification kit). Then, 10X serial dilution were prepared for GAPDH and CD151 to generate standard curves in qRT-PCR.

Real-time RT-PCR (qRT-PCR)

Real-time RT-PCR was performed using LightCycler® LC480 Probe Master (Roche Diagnostics). Probes for CD151 (cat. no.: 04688660001) and housekeeping gene, GAPDH (cat. no.: 04688589001) were purchased from Universal ProbeLibrary (Roche Diagnostics) and the primers were synthesized from Bioneer. The primer sequences were CD151-F: 5'-CTG CGC CTG TAC TTC ATC G-3' and CD151-R: 5'-TTC TCC TTG AGC TCC GTG TT-3'; GAPDH-F: 5'-CTC TGC TCC TCC TGT TCG AC-3' and GAPDH-R: 5'-ACG ACC AAA TCC

GTT GAC TC-3'. qRT-PCR was carried out in 20µL volumes containing 4µg of cDNA, 1X LightCycler® LC480 Probe Master, 2µL of probes and 0.8–1.0pmol of each primer. qRT-PCR was carried out on LightCycler® LC480 using a 96 well plate (Roche Diagnostics). All reactions were run in duplicates. The amplification program consisted of pre-incubation at 95°C with a 10min hold, denaturation at 95°C with a 10s hold, followed by annealing at 54°C with a 10s hold (40 cycles) and extension at 72°C with a 1s hold. The sizes of the amplicons were 107 bp and 112 bp for *CD151* and *GAPDH*, respectively.

Statistical analysis

Standard curves for GAPDH and CD151 were optimized by using diluted PCR products. Gene expressions of each sample were obtained by comparing the crossing points (Ct) with the standard curve. The obtained gene expression or concentration of CD151 gene was then normalized with GAPDH. Mean expressions of CD151 for each case were grouped according to the ER status, PR status, c-erbB-2 expression and tumour grades. The significant association of CD151 expression with the ER status, PR status and c-erbB-2 expression were determined by *t*-test unequal variances while the relation of CD151 and tumour status was determined by ANOVA.

RESULTS

In our study, PCR efficiencies for CD151 and GAPDH were 1.674 and 1.758, respectively. The normalized mRNA expressions of CD151 for all the samples examined in this study are listed in Table 1. The mean CD151 expression in different histological groups are illustrated in Figure 1.

Our preliminary real time RT-PCR results revealed that there was significant difference between CD151 expression and the ER status (p=0.012) and PR status (p=0.009) of breast tumour. Higher CD151 mRNA level was detected in ER positive and PR positive breast tumours. Conversely, CD151 expression was not associated with the tumour grade (p=0.057) and *c-erbB-2* expression (p=0.060) in our present study.

DISCUSSION

Several members of the tetraspanin superfamily have been identified as metastasis suppressor genes in cancer pathways. The CD151 gene was reported to be the first member of the tetraspanin superfamily, which plays a crucial role as promoter in cancer metastasis (Testa et al. 1999). Notably, CD151 gene mediates cell migration and facilitates invasion by regulating laminin (LN)-binding intergrins such as α3β1, α6β1, α6β4, and α7β1 through formation of CD151-integrin complexes called tetraspanin-enriched microdomains (TEM) (Liu et al. 2007). Recent study on knockdown CD151 expression in breast cancer cell showed that hepatocyte growth factor (HGF) receptor/c-met signaling pathway might be changed by the decreased Akt phosphorylation in cells lacking CD151 (Klosek et al. 2009). C-met signaling pathway plays a crucial role in mitogenic, proliferative, morphogenic and angiogenic activities. It correlates with breast cancer progression and metastasis (Park et al. 2005). The ability of CD151 in mediating cell migration suggested that CD151 is possibly involved in a molecular mechanism that could lead to metastatic progression of cancerous cells (Hashida et al. 2003). This hypothesis is proven by Novitskaya et al. (2010) and Sadej et al. (2010). They reported that CD151 expression is increased in invasive breast carcinomas and higher tumour grade and node metastasis. Additionally, CD151 is confirmed as a

Table 1: Clinicopathologic information for 45 breast cancer patients

Patient ID	Age	Tumour Grade	HPE Diagnosis*	Lymph Node Status	ER† Status	PR† Status	c-erb-2† Status	CD151 Concentration
26	42	1	IDC	-	+	+	-	0.25
43	37	2	Mucinous carcinoma	+	+	+	-	0.36
51	79	2	Mixed mucinous	+	+	+	-	0.32
57	52	3	IDC	+	+	+	+	0.46
86	68	3	DCIS	+	+	+	-	0.54
87	58	1	IDC	-	+	+	-	0.49
112	53	2	DCIS	-	+	+	-	0.95
113	30	3	IDC	+	-	-	-	0.03
117	46	2	IDC	+	-	+	-	0.35
122	53	1	IDC	+	-	-	+	0.23
125	75	3	IDC	-	+	+	-	0.40
126	52	2	IDC	+	+	-	+	0.22
127	22	1	DC	-	+	+	+	0.25
138	64	2	IDC	-	-	+	-	0.20
142	48	3	IDC	+	+	+	-	0.30
147	41	2	IDC	+	-	-	-	0.07
151	53	2	IDC	+	+	-	+	0.24
152	59	1	IDC	+	-	+	+	0.16
153	43	2	IDC	-	-	-	+	0.28
155	49	1	Lobular carcinoma	-	+	+	+	0.33
156	31	3	IDC	+	-	+	-	0.31
158	45	3	IDC	+	-	+	-	0.21
159	60	1	IDC	-	+	+	-	0.28
160	31	3	IDC	+	+	+	+	0.07
163	45	2	IDC	-	+	+	-	0.40
164	43	2	IDC	-	-	-	-	0.21
165	47	2	IDC	-	+	+	+	0.10
168	58	2	IDC	+	-	-	-	0.10
170	52	2	IDC	-	-	-	+	0.13
171	57	2	IDC	+	+	+	+	0.09
172	65	2	IDC	-	+	+	-	0.23
173	43	2	IDC	-	-	-	-	0.30
174	57	2	IDC	+	-	-	+	0.23
177	58	2	IDC	-	+	+	-	0.57
179	47	2	IDC	+	+	-	-	0.14
180	47	3	IDC	+	+	+	-	0.15
181	36	3	IDC	+	+	+	+	0.26
183	47	2	IDC	+	-	+	+	0.13
185	60	2	IDC	+	+	-	+	0.35
186	54	3	IDC	-	-	-	+	0.06
188	44	1	IDC with DCIS	+	+	+	-	0.19
190	54	2	IDC	-	-	-	+	0.24
191	70	2	IDC	-	+	+	-	0.11
192	56	3	IDC	+	+	-	+	0.16
194	57	1	IDC	+	-	-	+	0.23

* HPE (histopathological examination): IDC (infiltrating ductal carcinoma); DCIS (ductal carcinoma *in situ*)
† ER (estrogen receptor), PR (progesterone receptor): + (positive); - (negative)

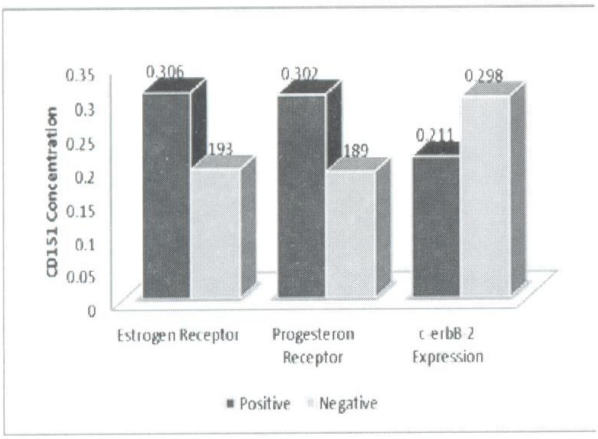

Figure 1: Mean CD151 concentration in different histological features (ER, PR and c-erbB-2) of breast cancer.

prognostic marker of outcome in lung and prostate cancers (Tokuhara et al. 2001; Ang et al. 2004).

Although previous studies have confirmed the ability of CD151 in facilitating cell migration and spreading, very limited information is available on the relationship between CD151 expression and hormone profile such as estrogen receptor and progesterone receptor status in breast cancer patients. The present study revealed that there was significant association between CD151 expression and ER positive ($p<0.05$) and PR positive subtypes ($p<0.01$). CD151 expression was higher in ER positive compared to ER negative breast tumours. It is also expressed at a higher level in PR positive relative to the PR negative breast tumours. Since ER positive breast cancer patients have better survival than ER negative patients, we suggest that the expression of CD151 could be another predictor for a better survival in breast cancer patients. Our findings is contrary with previous findings by Tokuhara et al. (2001), Ang et al. (2004) and Zijlstra et al. (2008) which said that CD151 expression is increased in advanced stage of the disease and shorter overall survival. However, our result is consistent with the findings reported by Voss et al. (2011). Voss et al. (2011) postulated that CD151 expression may prevent the transition of endometrial cancer to a more aggressive phenotype. They found that high CD151 expression is correlated with improved survival in the patients. How CD151 prevent the tumour cell transition is unknown. The pathway or biochemical function of CD151 in estrogen dependent breast cancer is still unknown and remains to be investigated.

Yang et al. (2008) had suggested possible connection between high CD151 transcript level and c-erbB-2 expression in breast tumour (Yang et al. 2008). Our study revealed that there is no significant difference between CD151 expression and c-erbB-2 expression. Nevertheless, our findings revealed lower CD151 mRNA level in triple negative (ER, PR and c-erbB-2 negative) breast carcinomas. The average CD151 expression level in triple negative breast tumours (113, 147, 164, 168 and 173) is 0.14, which is approximately one fold lower that other types of tumours (0.27). This observation suggests the important role of CD151 in the pathogenesis of triple negative breast tumours. Thus, c-erbB-2 expression may not link directly with CD151 expression but its expression depends on ER and PR status as well.

Several research teams have found that elevated CD151 mRNA level is associated with a more advanced and aggressive stage of the disease. In prostate cancer, CD151 expression was significantly different among well, moderately and poorly differentiated tumours (Hashida et al. 2003; Ang et al. 2004). However, CD151 expression is not correlated with breast tumour grades in our study ($p<0.05$). Our result is unexpected. The reason for inconsistency of our result with previous findings could be due to the sample size in our study. More than 70% of the subjects used in this study were grade 1 and 2 breast tumours (N=33/45).

Our results showed that elevated CD151 expression may be associated

with the hormonal status of breast cancer patients, mainly ER positive and PR positive breast cancers. Approximately 70% of ER positive breast cancer patients respond to hormonal therapy such as tamoxifen. For unknown reasons, the remaining 30% of ER positive breast cancer patients are resistant to hormonal therapy (Parikh et al. 2005). This observation suggested that ER status alone is not sufficient to determine the hormone responsiveness in breast cancer patients.

Our results showed that CD151 could be another important prognostic marker and treatment decision in estrogen dependent breast cancer patients. Extensive study of CD151 mRNA level in estrogen-dependent breast tumours is crucial to delineate its role in breast cancer pathogenesis.

Our study however has limitations due to the inter-observer variability of the pathologists in the histopathology examination of ER, PR, c-erbB-2 and tumour grade. Therefore, larger sample size is needed to verify the correlation between CD151 expression level and ER and PR status. Alternatively, the role of CD151 gene could be determined by studying the function of the gene in breast cancer cell line.

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REFERENCES

Ang, J., Lijovic, M., Ashman, L.K., Kan, K. & Frauman, A.G. 2004. CD151 protein expression predicts the clinical outcome of low-grade primary prostate cancer better than histologic grading: A new prognostic indicator? *Cancer Epidemiol Biomarkers Prev* **13**: 1717–1721.

Hashida, H., Takabayashi, A., Tokuhara, T., Hattori, N., Taki, T., Hasegawa, H., Satoh, S., Kobayashi, N., Yamaoka, Y. & Miyake, M. 2003. Clinical significance of transmembrane 4 superfamily in colon cancer. *Br J Cancer* **89**: 158–167.

Klosek, S.K., Nakashiro, K., Hara, S., Goda, H., Hasegawa, H. & Hamakawa, H. 2009. CD151 regulates HGF-stimulated morphogenesis of human breast cancer cells. *Biochem Biophys Res* **379**(4): 1097-1100.

Kohno, M., Hasegawa, H., Miyake, M., Yamamoto, T. & Fujita, S. 2002. CD151 enhances cell motility and metastasis of cancer cells in the presence of focal adhesion kinase. *Int J Cancer* **97**: 336-343.

Liu, L., He, B., Liu, W.M., Zhou, D., Cox, J.V. & Zhang, X.A. 2007. Tetraspanin CD151 promotes cell migration by regulating integrin trafficking. *JBC* **282**(43): 31631-31642.

Novitskaya, V., Romanska, H., Dawoud, M., Jones, J.L. & Berditchevski, F. 2010. Tetraspanin CD151 regulates growth of mammary epithelial cells in three-dimensional extracellular matrix: implication for mammary ductal carcinoma in situ. *Cancer Res* **70**: 4698-4708.

Parikh, P., Palazzo, J.P., Rose, L.J., Daskalakis, C. & Weigel, R.J. 2005. GATA-3 expression as a predictor of hormone response in breast cancer. *JACS* **200**(5): 705-709.

Park, C.H., Lee, M.H., Chang, E.S., Song, B.J. & Jung, S.S. 2005. Expression of c-met gene in invasive ductal carcinomas. *Breast Cancer Research* **7**: P12.

Sadej, R., Romanska, H., Kavanagh, D., Baldwin, G., Takahashi, T., Kalia, N. & Berditchevski, F. 2010. Tetraspanin CD151 regulates transforming growth factor beta signaling: implication in tumour metastasis. *Cancer Res* **70**: 6059-6070.

Testa, J.E., Brooks, P.C., Lin, J.M. & Quigley, J.P. 1999. Eukaryotic expression cloning with an antimetastatic monoclonal antibody identifies a tetraspanin (PETA-3/CD151) as an effector of human tumour cell migration and metastasis. *Cancer Res* **59**: 3812-3820.

Tokuhara, T., Hasegawa, H., Hattori, N., Ishida, H., Taki, T., Tachibana, S., Sasaki, S. & Miyake, M. 2001. Clinical significance of *CD151* gene expression in non-small cell lung cancer. *Clin Cancer Res* **7**: 4109–4114.

Voss, M.A., Gordon, N., Maloney, S., Ganesan, R., Ludeman, L., McCarthy, K., Gornall, R.,

Schaller, G., Wei, W., Berditchevski, F. & Sundar, S. 2011. Tetraspanin CD151 is a novel prognostic marker in poor outcome endometrial cancer. *British J of Cancer* **2011**: 1-8.

Yang, X.H., Richardson, A.L., Torres-Arzayus, M.I., Zhou, P., Sharma, C., Kazarov, A.R., Andzelm, M.M., Strominger, J.L., Brown, M. & Hemler, M.E. 2008. CD151 accelerates breast cancer by regulating α_6 integrin function, signaling, and molecular organization. *Cancer Res* **68**: 3204-3213.

Yauch, R.L., Berditchevski, F., Harler, M.B., Reichner, J. & Hemler, M.E. 1998. Highly stoichiometric, stable, and specific association of integrin α3β1 with CD151 provides a major link to phosphatidylinositol 4-kinase, and may regulate cell migration. *Mol Biol Cell* **9**: 2751-2765.

Zijlstra, A., Lewis, J., Degryse, B., Stuhlmann, H. & Quigley, J.P. 2008. The inhibition of tumour cell intravasation and subsequent metastasis via regulation of in vitro tumour cell motility by the tetraspanin CD151. *Cancer Cell* **13**: 221-234.