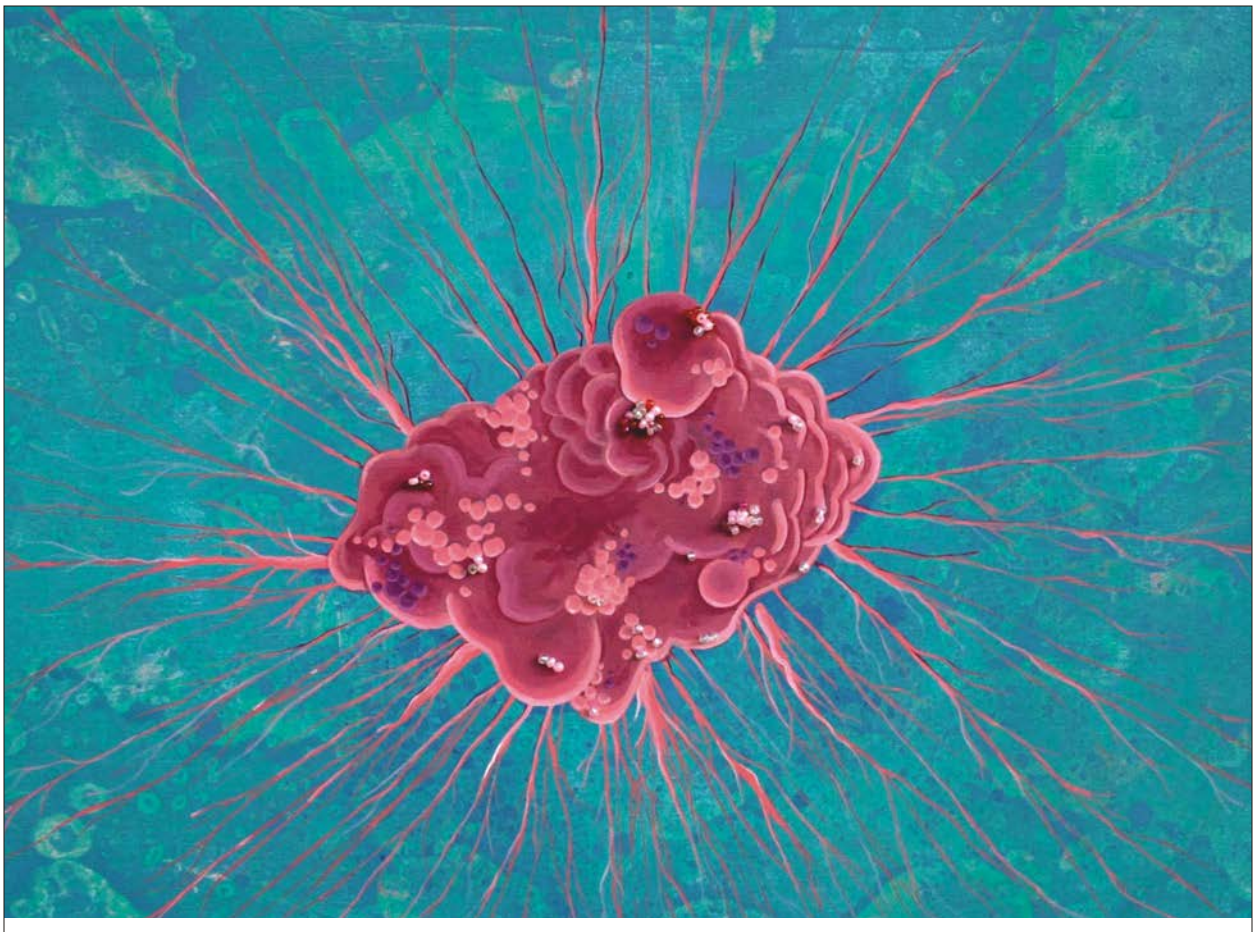

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# Immunohistological Localization of Human *FAT1* (*hFAT*) Protein in 326 Breast Cancers. Does this Adhesion Molecule Have a Role in Pathogenesis?

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**Abstract:** The immunohistological localization of human *FAT1* (*hFAT*) protein, a recently described member of the cadherin superfamily, was evaluated in 326 breast cancers and correlated with histologic type and grade. All tumors showed diffuse staining for *hFAT*. Immunoexpression of the protein was generally strong in Lobular Carcinoma *in situ* (LCIS, n = 2) and Ductal Carcinoma *in situ* (DCIS, n = 55). *hFAT* was also strongly immunoexpressed in all types of invasive carcinoma. Grade 3 DCIS displayed the highest *hFAT* intensity compared to lower grade tumors, with significant differences between grade 1 and 3 ($p = 0.015$) and grade 2 and 3 ($p = 0.047$). With invasive Ductal Carcinomas (n = 128), the difference was not as clear-cut as most tumors showed moderate (n = 63) or strong staining (n = 49), although grade 3 IDC revealed significantly decreased immunoexpression compared to grade 1 IDC ($p = 0.03$). These results illustrate that *hFAT1* does not display the pattern of expression seen with the E-cadherin-β-catenin adhesion complex; however, its overexpression and diffuse expression in both *in situ* and invasive carcinoma strongly suggests a role in carcinogenesis. From the known functions of *FAT1* it is suggested that the concurrent loss of classical cadherins from cell-cell junctions accompanied by increased *FAT1* expression contributes to loss of duct formation and increased cell migration and invasion.

**Key words:** *hFAT*, immunohistochemistry, breast cancer, adhesion proteins

## Introduction

*FAT1* is a recently categorized member of the cadherin-superfamily, named such because of its molecule resembles the cadherin-like protein encoded by the *FAT* gene, which functions as tumor suppressor in *Drosophila* (Mahoney *et al*., 1991). The first human *FAT* encoding gene, named *FAT1* (*hFAT*) was localized to 4q34-q35 (Dunne *et al*., 1995). The extracellular domain of *hFAT* comprises 4594 residues with 34 tandem cadherin repeats (cad-rpts), five epidermal growth factor-like repeats (EGF-rpts) and a laminin A-G domain (LAG-rpts). The cytoplasmic domain contains two blocks of protein residues of 137 and 84 amino acids that incorporate a region weakly homologous to the cadherin catenin-binding region. In an *in situ* hybridization study (Dunne *et al*., 1995) high levels of *hFAT* mRNA were detected in fetal epithelium and the epithelium of adult small and large intestines. Other adult tissues such as pancreas, kidney, lung, ducts of salivary glands and squamous epithelium of tonsillar crypts generally showed low levels of *hFAT* mRNA expression. Among adult neoplasms,

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the highest level of *hFAT* mRNA was detected in a hyperplastic colonic polyp, whereas varying signal strengths were found in colorectal and breast carcinoma, with only focal expression in pulmonary oat cell carcinoma and malignant lymphoma (Dunne *et al*., 1995).

The extracellular domains of classical cadherins are known to form parallel dimers and to mediate calcium-dependent homophilic interaction. The intracellular domains interact with α- and β-catenin, which anchor the adhesion complex to the actin cytoskeleton, these complex structures contributing strong intercellular adhesiveness (Yap *et al*., 1997). Many studies have been performed of individual members of cadherin superfamily. Loss of membranous E-cadherin was found to be inversely correlated with high grade breast carcinoma and number of mitosis (Gamallo *et al*., 1993) and was associated with invasiveness (Siitonen *et al*., 1996; Sormuen *et al*., 1999). In contrast, overexpression of N-cadherin induced cell migration, invasion and metastasis (Nieman *et al*., 1999; Hazan *et al*., 2000).

In *Drosophila,* recessive (loss of function) mutations of the *FAT* gene led to overgrowth of larval imaginal disc epithelial cells in the wing bud, indicating a role in the control of cell proliferation and morphogenesis (Bryant *et al*., 1988). Having been identified only relatively recently, the function of *FAT1 in vivo* in humans has yet to be reported. *In vitro* studies indicate that mammalian *FAT1* regulates the actin cytoskeleton to modulate cell contacts and polarity and may play an integrative role in regulating cell migration (Moeller *et al*., 2004; Tanoue and Takeichi, 2005). These characteristics suggest that *FAT1* may be important in cancer metastasis, a notion substantiated by the increased expression of *FAT1* mRNA in adult neoplastic cells (Dunne *et al*., 1995).

In the present study, we utilized affinity-purified specific rabbit-anti-*FAT1* antibodies to examine *hFAT* expression in a large number of *in situ* and invasive types of breast cancer, in the anticipation that its localization and intensity of immunoreactivity may indicate its major function in human neoplastic cells.

## Materials and Methods

Three hundred and twenty six cases representing a broad spectrum of histologic subtypes of breast cancer from the year 2000 were retrieved from the files of the Hunter Area Pathology Service, Newcastle, Australia. All specimens had been fixed in 10% buffered formalin and routinely processed to paraffin. The histological type and grade of carcinoma were reported according to the World Health Organization classification (Tavasoli and Devilee, 2003) and the Nottingham modification of the Bloom-Richardson grading system, respectively (Elston and Ellis, 1991). Grading of DCIS was performed according to the WHO criteria (Tavasoli and Devilee, 2003).

Ductal carcinoma was divided into three groups, namely, Ductal Carcinoma *in situ* (DCIS), Infiltrating Ductal Carcinoma (IDC) and IDC with Extensive Intraductal Component (EIC). The latter was defined as the presence of more than 25% of DCIS or the presence of DCIS beyond the edges of the invasive carcinoma (Connoly *et al*., 1998). This separate group was included to allow the comparison of *hFAT* immunostaining of *in situ* carcinoma with the concurrent invasive component.

### *Fat1 Antibody Production*

Affinity-purified rabbit antibodies specific for the cytoplasmic region of human *FAT1* were prepared using a GST-*FAT1* fusion protein as immunogen. In brief, the 5' end of the *FAT1* cDNA (amino acids 4422 to the end) was ligated into the EcoR1 site of the pGEX1 vector (Amersham

Pharmacia Biotech, Uppsala, Sweden). The construct was sequenced to ensure that the coding sequence for the fusion proteins was in frame, then transformed into *E. coli* strain JM109 for IPTG- (isopropyl β-D-thiogalactosidase) induced protein production by standard methods. Soluble proteins from the sonicated bacteria were affinity purified over Glutathione Sepharose 4B (Pharmacia Biotech, Uppsala, Sweden) and eluted with buffer containing 15 mM glutathione. The eluted fusion protein was quantified by the Bradford dye-binding assay (BioRad, Hercules, CA, USA) and checked for purity by SDS-PAGE analysis and silver staining. Antibodies were raised by subcutaneous injection of 0.2 mg of purified recombinant antigen in Freund's complete adjuvant (Difco, MI, USA) into a rabbit. Following repeated injections of antigen in incomplete adjuvant, antiserum was collected and purified by affinity chromatography against GST-*FAT1* fusion protein. Non-specific antibodies were removed by incubation with non-fused GST protein.

The specificity of the antibodies was confirmed in 3 ways:

- To confirm immunoreactivity, rabbit kidney (RK13) cells were transfected with an expression construct incorporating the cytoplasmic domain of *FAT1* together with a FLAG epitope and an IL-3 signal peptide. Two-colour immunofluorescent staining demonstrated coincidence of staining for FLAG with a mouse monoclonal antibody (M2) and the anti-*FAT1* antibodies in the transfected cells. In addition, immunoprecipitation and blotting experiments showed that from lysates of these cells both the M2 and anti-*FAT1* precipitated and blotted the same band.
- Endogenous *hFAT1* was identified by immunoblotting C32 melanoma cells when a band at ≥ 500 kDa was labeled with the anti-*FAT1* antibodies but not with pre-immune antibodies. The surface nature of this *FAT1* band was shown by labeling the cells with biotin and demonstrating that the band precipitated with the anti-*FAT1* antibodies bound streptavidin.
- Immunostaining of endogenous *FAT1* was ablated by pre-absorption of the antibody with GST-*FAT1* fusion protein but was essentially unaltered by pre-absorption with an irrelevant GST-fusion protein.

*Immunohistochemistry*

Five-micron sections were mounted on silane-coated slides, deparaffinised in xylene, rehydrated in graded alcohols and rinsed in tap water for 5 min. Endogenous peroxidase activity was blocked in 3% hydrogen peroxide in methanol for 20 min, following which the sections were washed in phosphate buffered saline (PBS) for 5 min. Antigen retrieval was conducted by heating in a microwave pressure cooker in citrate/EDTA buffer at pH 8.0 in a 1000 W microwave oven for 10 min. The sections were allowed to cool to room temperature in the buffer for 20 min before washing in PBS for 5 min. Blocking was performed with normal blocking serum (VECTASTAIN Universal Elite ABC Kit) for 20 min before overnight incubation with the primary anti-*FAT1* antiserum (1:5,000) at 4°C before staining with an avidin biotin system (VECTASTAIN Elite ABC kit) with diaminobenzidine as the chromogen and a light Mayer's hematoxylin as counterstain.

Staining intensity was graded on a scale of 0 to 3, where, 0 = no staining, 1 = weak, 2 = moderate and 3 = strong. Initially, the membrane localization of antigen was searched for but this was not continued when all positive staining was found to be located to the cytoplasm.

Correlation between intensity of *hFAT* expression and histologic grade in ductal carcinomas was evaluated by the Chi-square test or Fisher's exact test. $p \leq 0.015$ were considered statistically significant.

## Results

Immunoreactivity for *hFAT* was localized only to the cytoplasm of both neoplastic and non-neoplastic mammary epithelia. Neither membrane or nuclear localization of the immunoreactivity was observed in epithelial cells. In non-neoplastic lobules, apocrine metaplasia and ductal epithelial hyperplasia, strong immunostaining was generally displayed (Fig. 1a and b) compared to that in normal ductal epithelium. Antigen concentration was strongest at the apices of the cells with some localization at the cell bases. Myoepithelial cells displayed greater intensity of staining for *hFAT* compared to epithelial cells. Vascular smooth muscle, stromal fibroblasts and infiltrating lymphocytes also displayed immunoreactivity but generally of weak or moderate intensity.

Initially, a scoring system incorporating the percentage of positive-staining tumor cells and the intensity of staining was applied, but this was found not to be contributory as the cytoplasmic antigen when present, was expressed homogenously in more than 75% of cells within the same tumor with no specific localization (Fig. 2a and b and Table 1). Only scattered cells displayed different, generally greater stain intensity (Fig. 2a). Staining was of a granular nature, most evident in strongly expressing tumor cells that displayed coarse granularity (Fig. 2b).

*Ductal Carcinoma in situ (DCIS)*

The staining in DCIS was of moderate to strong intensity (Fig. 3), the majority (43/55; 78.18%) displaying strong immunoreactivity and only 1 tumor (1.8%) showing weak staining (Table 1). Intensity of staining correlated with higher histologic grades of DCIS with significant difference between grade 1 and 3 ($p = 0.015$) and grade 2 and 3 ($p = 0.047$). The difference in *hFAT* immunoexpression between grade 1 and 2 was not significant ($p = 0.581$) (Table 1).

*Infiltrating Ductal Carcinoma (IDC)*

The majority of tumors displayed moderate (65/137; 47.44% and Fig. 4) or strong (56/137; 40.88%; Fig. 4a and b) immunoreactivity (Table 2). In contrast to the findings in DCIS, higher histologic grades of infiltrating ductal carcinoma appeared to display reduced *hFAT*-staining intensity;

Table 1: *hFAT* immunoreactivity in 55 cases of Ductal Carcinoma *in situ* (DCIS)

| Stain score | 1 | 2 | 3 | Total |
|---|---|---|---|---|
| Grade 1 | 0 (0.00%) | 4 (44.44%) | 5 (55.56%) | 9 (100.00%) |
| Grade 2 | 1 (4.35%) | 6 (26.09%) | 16 (69.57%) | 23 (100.00%) |
| Grade 3 | 0 (0.00%) | 1 (4.35%) | 22 (95.65%) | 23 (100.00%) |
| Total | 1 (1.82%) | 11 (20.00%) | 43 (78.18%) | 55 (100.00%) |

Table 2: *hFAT* immunoreactivity in 137 cases of invasive ductal carcinoma (IDC)

| Stain score | 1 | 2 | 3 | Total |
|---|---|---|---|---|
| Grade 1 | 2 (5.00%) | 13 (32.50%) | 25 (62.50%) | 40 (100.00%) |
| Grade 2 | 9 (13.24%) | 35 (51.47%) | 246 (35.29%) | 68 (100.00%) |
| Grade 3 | 5 (17.24%) | 17 (58.62%) | 7 (24.15%) | 29 (100.00%) |
| Total | 16 (11.68%) | 65 (47.44%) | 56 (40.88%) | 137 (100.00%) |
| IDC with EIC | 5 (5.3%) | 38 (40.4%) | 51 (54.3%) | 94 (100.00%) |

Table 3: *hFAT* immunoreactivity in lobular carcinoma

| | Scoring of *hFAT* staining | | | |
|---|---|---|---|---|
| | 1 | 2 | 3 | Total |
| Lobular Carcinoma *in situ* (LCIS) | 0 (0.00%) | 0 (0.00%) | 2 (100.00%) | 2 (100.00%) |
| Invasive Lobular Carcinoma (ILC) | 0 (0.00%) | 12 (31.58%) | 26 (68.42%) | 38 (100.00%) |

Fig. 1(a): There is apical localization of *hFAT* in the mammary duct. Staining is cytoplasmic and granular. (b): Apocrine metaplasia showing strong granular cytoplasmic staining with apical accentuation

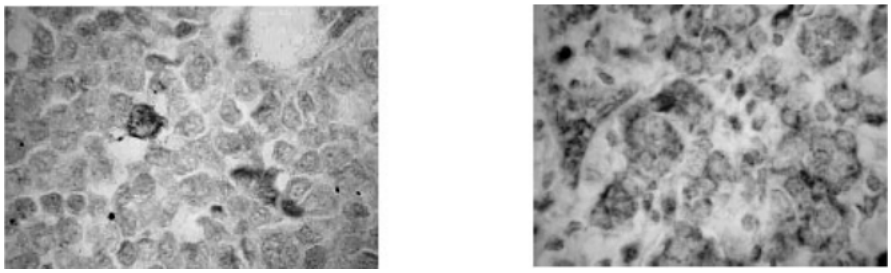

Fig. 2 (a): Weak staining in DCIS grade 2. Staining is homogenous throughout the tumor except for cattered strongly expressing tumor cells. (b) IDC grade 3 displaying the coarse ranularity associated with strongly expressing cells

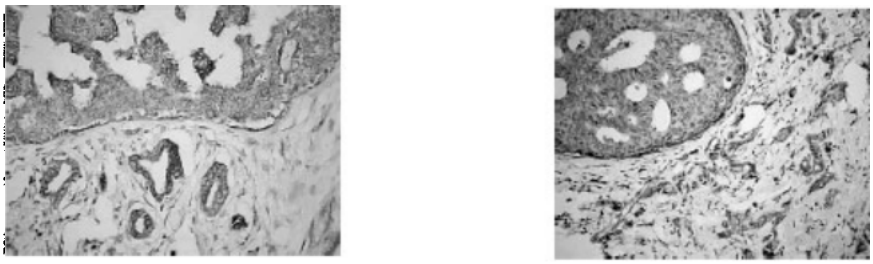

Fig. 3 (a): Moderate intensity staining in DCIS grade 1 with concurrent tubular carcinoma (IDC grade 1). (b) Strong staining in DCIS grade 1 with concurrent IDC grade 2

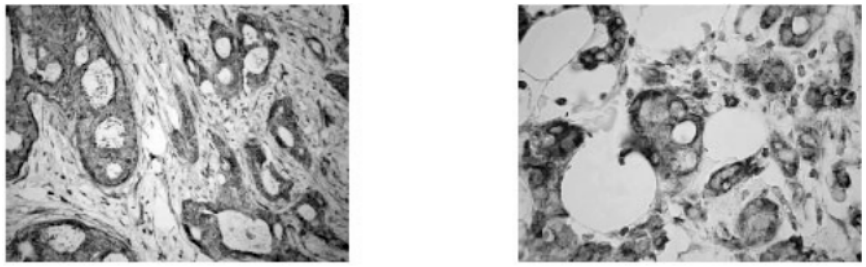

Fig. 4 (a): IDC grade 1 showing moderate staining. The duct with DCIS on the left displays strong staining in a peripheral rim of partially preserved myoepithelial cells . (b): IDC grade 3 showing strong granular cytoplasmic staining for *hFAT*

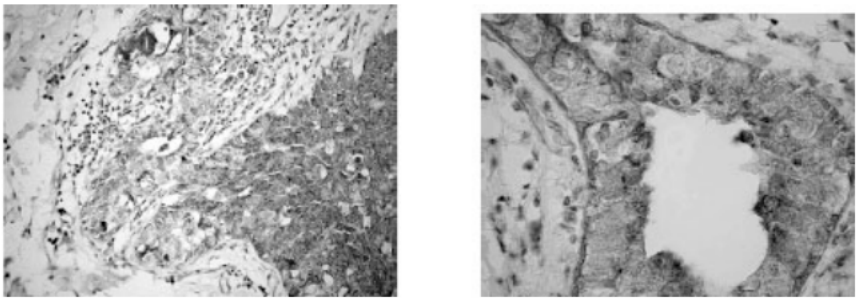

Fig. 5 (a): Invasive front of IDC grade 3 showing reduced immuno expression of *hFAT* compared with the main tumber. (b): Cancerization of duct by a weakly staining IDC showing the contrast between tumber cells and strongly staining residual duct epithelium

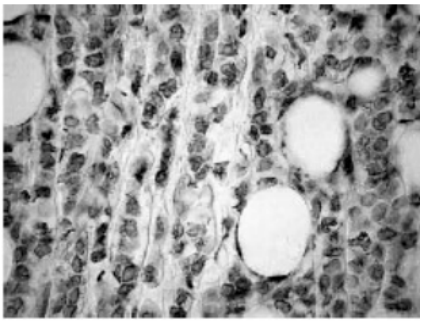

Fig. 6: Infiltrating lobular carcinoma displaying strong granular staining. Note the staining of a dipocytes in the field

however, *hFAT* staining was only significantly different between grade 1 and 3 ($p = 0.027$) with no significant difference between grade 1 and 2 ($p = 0.10$) and between grade 2 and 3 tumors ($p = 0.585$). Staining was generally homogenous in the cytoplasm but in strongly staining tumors the staining was clearly coarsely granular (Fig. 2b and 4b). This coarsely granular pattern of staining did not appear to correlate with histologic grade and was also seen in all strongly expressing cases of infiltrating lobular carcinoma. Interestingly, while reduced *hFAT* immunoexpression was observed at the invading front of tumors, it was only observed in two cases (Fig. 5a). In tumors showing weak staining, cancerized ducts displayed a contrast between tumor cells and strongly expressing residual ductal epithelium (Fig. 5b).

The cases of IDC with EIC also showed a pattern of staining not dissimilar to that seen in DCIS and IDC in that most tumors displayed moderate (38/94, 40.4%) to strong (51/94, 54.3%) staining (Table 2). In these tumors the intensity of stain in the DCIS component generally corresponded to the intensity expressed by the infiltrating carcinoma (Fig. 3a and b).

Among the IDCs were 6 cases of colloid/mucinous carcinoma, 83.33% (5/6) of which displayed strong *hFAT* immunoreactivity regardless of their histologic grade. The remaining one tumor showed moderate intensity staining. There were three tumors that were tubular carcinomas and two showed strong and one moderate intensity staining (Fig. 3a and b).

*Lobular Carcinoma*

Both Lobular Carcinoma *in situ* (LCIS) and Invasive Lobular Carcinoma (ILC) displayed the strongest immunoreactivity for *hFAT* compared with other tumour subtypes (Table 3). The two cases of pure LCIS in this study together with 68.42% (26/38) of ILCs displayed intensity scores of 3. Moderate staining intensity was found in the remaining 31.58% (12/38) of ILC. Among the ILCs with strong staining, were 72.73% (8/11) and 66.67% (18/27) cases subtyped as classical and pleomorphic ILC respectively. Moderately expressing ILC contained scattered single tumors cells that were strong expressing (Fig. 6).

## Discussion

The cadherin superfamily comprises several subfamilies that include classic cadherins, desmosomal cadherins, protocadherins, Flamingo/Celsr, Dachsous and *FAT* (Tanoun and Takeichi, 2005). Classic cadherins and desmosomal cadherins function as cell-cell adhesion molecules with a well-defined role in neoplasia (Wood and Leong, 2003) however, the primary biological function of the other cadherins is not established and their importance to neoplasia remains largely unexplored. *FAT* is the largest member of the family with 34 cadherin repeats in the extracellular region and is highly conserved from zebrafish to man (Tanoue and Takeichi, 2005; Down *et al*., 2005), but its gigantic size of around 500-600 kDa has made functional studies difficult. The original *FAT* gene discovered in *Drosophilia,* was shown to function as a tumour suppressor gene responsible for hyperplastic overgrowth of imaginal discs (Mahoney *et al*., 1991) and as a regulator of planar polarity (Ma *et al*., 2003). Subsequently, a second gene, *FAT*-like, was identified in *Drosophila* and shown to function as a spacer molecule responsible for the morphogenesis and maintenance of tubular structures of ectodermal origin (Castillejo-Lopez *et al*., 2004). Recently, in the worm *Celegans*, *FAT*-like was shown to be a key component for cell invasion through basement membranes during development (Sherwood *et al*., 2005). Mammals have now been shown to express 4 distinct *FAT* genes, *FAT1*, Fat 2, *FAT 3* and *FAT J* and together with the *drosophila* Fats, have been divided into 2 major subfamilies (Tanoue and Takeichi, 2005), namely, FAT and *FAT J* and *FAT*-like and *FAT 1, FAT 2* and *FAT 3.* Of these, only FAT 1 has been studied in any detail. *FAT1* null mice exhibited perinatal lethality from renal failure due to fusion of glomerular podocytes as a result of abnormal slit junction formation between these glomerular epithelial cells (Ciani *et al*., 2003). These findings substantiate a role of *FAT1* as a spacer molecule-analogous to *FAT*-like in drosophila-however, other low penetrance defects including failures in forebrain and eye development were recorded (Ciani *et al*., 2003), suggesting that in these *FAT1* null mice partial redundancy with *FAT 3* may have ameliorated the phenotype observed (Tanoue and Takeichi, 2005). *In vitro* studies of *FAT1* function indicate that it modulates epithelial cell-cell contacts and polarity and perhaps also cell migration, through Ena/VASP-mediated interaction with the actin cytoskeleton (Moeller *et al*., 2004; Tanoue and Takeichi, 2005).

In the only study of *FAT1* expression in human tissues by *in situ* hybridization, mRNA transcripts were largely restricted to embryonic tissues and to tumours in the adult (Dunne *et al*., 1995), but to date these findings have not been pursued. Our development of specific anti-*FAT1* antibodies enabled a more comprehensive analysis of the expression of distribution of *FAT1* in human cancers and this report represents the first study of *hFAT1* immunoexpression in a large number of breast cancers.

In an earlier study (Sormunen *et al*., 1999), we demonstrated that the E-cadherin-β catenin complex progressively breaks down in invasive ductal carcinoma compared to DCIS. Furthermore, the loss of membrane localization of these proteins and eventual loss from the cytoplasm correlated with increasing histologic grades of both DCIS and IDC, the findings supporting the concept that loss of this adhesion complex was associated with tumor progression and invasion. As *hFAT1* has been demonstrated to localize at both cell peripheries and cell-cell contact sites we surmised that the protein may demonstrate a similar correlation with histological grades of breast cancer as the E-cadherin-β catenin complex, but this was not found to be so. Present findings demonstrate that *hFAT1* is immunoexpressed by both neoplastic and non-neoplastic mammary epithelium. Expression appears to be upregulated in hyperplasia and neoplasia. *hFAT1* stained more intensely in invasive cancers of all histologic grades and types and immunoexpression was diffuse and did not display membrane localization or accumulation at cell-cell contact sites. While variable intensities of immunoexpression were found among different histologic grades, *hFAT1* generally showed strong expression in both ductal and lobular carcinoma *in situ* with significant differences between grade 1 and 3 and between grade 2 and 3 DCIS. Similarly, in invasive ductal and lobular carcinomas the protein expression was invariably moderate or strong, with a significant difference in immunoexpression between grade 1 and 3 tumors but not between grade 2 and 3 and grade 1 and 2 IDCs. Staining intensities were generally not different in DCIS and concurrent invasive carcinoma, a property that we observed previously for other biological markers in breast cancer (Leong *et al*., 2001). While it would seem unlikely that *hFAT1* is a determinant of prognosis or behavior, its clearly increased expression in hyperplasia and all forms of neoplastic transformation strongly indicates a role in carcinogenesis.

Strong immunoreactivity intensity of *hFAT* was revealed in lobular, mucinous and tubular carcinoma, regardless of their histologic grade or invasiveness and this was also present in hyperplastic epithelium and apocrine metaplasia. Furthermore, the preexisting *hFAT* was observed in infiltrating tumour cells and tumour emboli in lymphatic vessels. These observations indicate that *hFAT* could not have a role in suppressing tumour invasion but may be involved in cell migration and morphogenesis. These findings in human breast cancer are consistent with the perceived function of *FAT1* from animal studies. In *FAT1* null mice there was no evidence of proliferative abnormalities in tissues containing stem cell populations which normally contain abundant *FAT1* (Ciani *et al*., 2003), strengthening the concept that the *Drosophila* orthologue of *FAT1* is not *FAT* (which does function as a tumour suppressor gene)1 but *FAT*-like (which does not) (Castillejo-Lopez *et al*., 2004). In flies and worms *FAT*-like is implicated in tubular formation and in tissue invasion (castillego-Lopez *et al*., 2004 Sherwood *et al*., 2005) and *in vitro*, mammalian *FAT1* plays a role in directed cell migration (Tanoue and Takeichi, 2005, 2004). The findings from our present study suggest that the increased expression of *FAT1* seen in all of a range of human breast cancers may reflect acquisition of any or all of these functions as a mechanism towards tumour progression.

Just how *FAT1* might function in tumour progression is currently unknown. It is now well established that loss of the classical E-cadherin-β-catenin complex from cell-cell junctions correlates with breast tumour progression (Sormunen *et al*., 1999). In their *in vitro* study of *FAT1*, Tanoue and Takeichi (2004) found that inhibiting the homotypic adhesion between E-cadherin or P-cadherin by the use of blocking antibodies altered β-catenin distribution such that its expression became diffuse, rather than being situated at cell-cell junctions. Concurrently, they found that the distribution of *FAT1* at cell-cell junctions also became diffuse or was lost entirely following this treatment, indicating that, at this site, the classic cadherin system is required for the localization

of *FAT1*. It might be suggested, therefore, that the loss of classical cadherins that accompanies breast cancer progression serves to drive *FAT1* out of the junctional complexes further facilitating the degeneration of ductal formation. At the same time, the generally increased expression of *FAT1* that we have observed to occur throughout the range of breast cancers somehow promotes increased cell migration and tissue invasion. In this regard, it will be instructive in future studies to examine the relative expression and distribution of *FAT1* in conjunction with staining for the classic cadherin complex.

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