Loss of imprinting of IGF2 in fibroadenomas and phyllodes tumors of the breast

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Abstract. Loss of imprinting (LOI) of insulin-like growth factor 2 (IGF2) is thought to be implicated in the pathogenesis of some tumors by upregulating IGF2 mRNA but its role in the pathogenesis of fibroadenomas (FAs) and phyllodes tumors (PTs) of the breast is yet to be studied. LOI of IGF2 was investigated in 25 FAs and 17 PTs which were heterozygous for Apa I polymorphism, and was found to be present in 13 FAs and 12 PTs. IGF2 mRNA expression was more upregulated in FAs and PTs than in paired surrounding normal tissues and laser microdissection showed that IGF2 mRNA expression was significantly higher in the stromal than the epithelial cells. LOI was not associated with upregulation of IGF2 mRNA, nor were MED12 mutations and methylation status of the differentially methylated region 0 (DMR0) of IGF2. These results demonstrate that IGF2 mRNA expression is more upregulated in FAs and PTs than in normal tissues, especially in their stromal cells, but such an upregulation is not related to LOI of IGF2, and that hypomethylation of DMR0 is unlikely to be involved in induction of LOI.

Introduction

Fibroadenomas (FA) and phyllodes tumors (PT) are fibroepithelial tumors of the breast with stromal and epithelial components. It has been suggested that the insulin-like growth factor 2 (IGF2) gene is associated with the pathogenesis of breast fibroepithelial tumors (1). The IGF2 gene is located within the IGF2/H19 imprinted gene cluster on chromosome 11p15 and is expressed predominantly from the paternal allele (2-4). IGF2 promotes the growth and proliferation of cells in many different tissues (5) and is highly expressed in various types of tumors (6-8) including fibroepithelial tumors of the breast (1). As reported in studies of Wilms’ tumors, loss of IGF2 imprinting (LOI) manifested by biallelic gene expression results in IGF2 overexpression and subsequent tumor formations (9,10). Occurrence of LOI in almost all types of cancers such as colorectal cancer (33-88%) (11-13), prostate cancer (83%) (14) and breast cancer (0-60%) (15-18) has been frequently reported, but the association of LOI with IGF2 expression has not been clearly established yet.

Imprinted genes are associated with CpG-rich regions which have allele-specific DNA methylation and are known as differentially methylated regions (DMRs) (15). Loss of methylation at the human IGF2 DMR0 has been reported to be associated with IGF2 LOI in several malignancies (2,6,19,20) including breast tumors (15,21). DMR0 methylation levels are significantly lower in cancer tissues (29-31%) than in normal tissues (45-51%) (6,15,20), but IGF2 mRNA expression is not always related to LOI or DMR0 methylation status (7,15,17), suggesting that aberrant IGF2 expression may well be induced by other unknown mechanisms.

MED12 mutations have recently been identified in fibroepithelial tumors of the breast (22), that is, in 47-59% of FAs and 67-80% of PTs (22-25) and were detected specifically in the stromal cells of these tumors. MED12 mutations were first described in a study of leiomyomas of the uterus, and enhanced expression of IGF2 has been found in leiomyomas with MED12 mutations (26), implicating that MED12 mutation is associated with IGF2 expression also in fibroepithelial tumors of the breast.

In the present study, we therefore first investigated whether LOI occurs in FAs and PTs and is associated with IGF2 expression since these important issues have not been properly addressed yet. Furthermore, we studied the impact of DMR0 methylation or MED12 mutation on LOI or IGF2 expression, respectively.

Materials and methods

Patient samples. For this study 58 FAs and 27 PTs from 52 and 24 female patients, respectively, were analyzed. Clinicopathological characteristics of these tumors are shown in Table I. All these tumors are the same as those analyzed...
in our previous study on MED12 mutations (23). The patients underwent tumorectomy or mastectomy between 1993 and 2005. Five patients had synchronous multiple FAs, one patient synchronous FA and PT, one patient metachronous FA and PT, and two patients metachronous multiple PTs. Histological diagnosis of all tumors was confirmed by two pathologists (E.M. and J.I.). This study was approved by the Osaka University Research Ethics Committee.

DNA extraction from formalin-fixed paraffin-embedded (FFPE) tumor tissues. For DNA extraction, three to eight 10-µm sections per tumor were cut from the FFPE tumor tissues and mounted onto a polyethylene naphthalate (PEN) membrane slide (Leica Microsystems GmbH, Wetzlar, Germany). The FFPE tumor tissues slides were stained with hematoxylin after deparaffinization and the tumor area was macrodissected with a scalpel and with stereoscopic assistance. Genomic DNA from the paraffin sections was extracted and purified using the QIAamp DNA FFPE kit (Qiagen, Valencia, CA, USA) and 1 µg of genomic DNA was subjected to sodium bisulfite treatment with the EpiTect Bisulfite kit (Qiagen).

Quantitative IGF2 DMR0 methylation analysis using NGS. Since it has been reported that IGF2 LOI correlates with hypomethylation of three CpGs (CpG 15-17) included in DMR0, we performed target sequencing of this region by means of next-generation sequencing (NGS). For allele-specific pyrosequencing, two primer sets were designed, which recognized the A or G allele of the rs3741210 polymorphism near the three targeted CpGs. The following NGS primers were designed for IGF2 DMR0 allele-specific methylation: forward 5′-GGGCCCCAGAAAAGCCACTGGACACACAG-3′, reverse for A-allele 5′-CAGGGTGGTGTCTGTGGGAGGGGGTTCCCAT-3′ and reverse for G-allele 5′-CAGGTTGGTGTCTGTGAGGAGGGGTTCCCAT-3′ (amplicon size = 111 bp; Fig. 1). A total volume of 30 µl contained 4 µl of bisulfite DNA and 0.4 µM of each primer. PCR was carried out using Takara Ex Taq® Hot Start Version (Takara Bio Inc., Shiga, Japan). The incubation was as follows: 37˚C for 10 min; 40 cycles at 95˚C for 30 sec, at 61˚C for 30 sec, at 72˚C for 30 sec, and a final extension at 72˚C for 10 min. Five microliters of each of the PCR products was digested with 15 units of Apa I at 37˚C for 60 min. The A allele (not digested by Apa I) produced 129 bp and the G allele (digested by Apa I) produced 80 and 49 bp. Samples which showed heterozygous A/G at the Apa I polymorphism were defined as informative and selected for RNA isolation.

RNA extraction from FFPE tissues. For RNA extraction, two to four 10-µm sections were removed from the FFPE tumor tissues. After deparaffinization, staining and macrodissection as detailed above, RNA was extracted and purified with the RNeasy FFPE kit (Qiagen). Reverse transcription from RNA to cDNA was performed with the ReverTra Ace qPCR RT kit (Toyobo, Osaka, Japan). The incubation was as follows: 37˚C for 20 min; 98˚C for 5 min.

Analysis of IGF2 imprinting status. RT-PCR was used to analyze RNAs from the informative specimens from 25 FAs and 17 PTs for allele-specific Apa I site polymorphism. PCR and Apa I digestion were performed as described above. A specimen was identified as LOI when two bands were clearly visible on a gel with a ratio of at least 1:4 as previously described (Fig. 2) (15).

Real-time RT-PCR. Quantitative mRNA expression was measured by using the Light cycler 480 Real-time PCR System (Roche Applied Science, Mannheim, Germany) at 95˚C (10 min), followed by 50 cycles at 95˚C (15 sec) and at 60˚C (60 sec), and finally one cycle at 50˚C (10 sec). IGF2 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) TaqMan® Gene Expression Assays (assay identification numbers: Hs01005963_m1 and Hs02758991_g1; Applied Biosystems, Foster City, CA, USA) were used for the real-time qPCR assay. The expression of IGF2 was normalized to that of GAPDH, and each assay was performed in duplicate.

IGF2 expression analysis using laser microdissection (LMD). A 10-µm section was cut from each of the FFPE tumor tissues of three FAs and three PTs and mounted onto a polyethylene naphthalate (PEN) membrane slide (Leica Microsystems GmbH), which was stained with hematoxylin after deparaffinization. One area of the epithelium or stroma was selected and dissected with the laser microdissection system LMD7000 (Leica). Each LMD specimen was automatically collected by gravity into the cap of a microdissection tube. The LMD specimens were then subjected to RNA extraction with the RNeasy FFPE kit (Qiagen). Reverse transcription, IGF2 imprinting analysis and real-time RT-PCR were performed as described above.
IGF2 expression analysis of matched normal breast tissues. Twelve normal breast tissues adjacent to the tumors could be obtained for six FAs and six PTs. These tissues were macrodissected and RNA was extracted with the RNeasy FFPE kit. Reverse transcription, IGF2 imprinting analysis and real-time RT-PCR were performed as described above.

Correlation with IGF2 expression and MED12 mutation status. The 23 FAs and 17 PTs which were identified as informative for Apa I polymorphisms were subjected to MED12 mutation analysis. Since mutation in the MED12 exon 2 reportedly correlates with IGF2 overexpression, we used NGS for target sequencing of this exon. In brief, DNA from the paraffin sections was amplified by means of PCR using the following primers: forward 5'-ACA ACTAA ACGCCGCT TTC-3' and reverse 5'-ATGCTCATCCCAAGAGACAG-3'; amplicon, 98 bp. Primers were designed to cover 93% of MED12 mutations of FAs as previously reported by Lim et al (22). The PCR amplicons were purified with NucleoSpin Gel and PCR Clean-Up (Macherey-Nagel, Düren, Germany). NGS was performed with the GS Junior system and the data were analyzed with GS AVA software, which can detect mutations with a minimum read count of two and a minimum read percentage of 0.25%. Variant allele frequencies were
calculated for each position and a threshold of 5% was used to characterize candidate variants based on the findings by Lim et al. (22).

Statistics. Associations of the clinicopathological characteristics with IGF2 imprinting status were evaluated by means of the chi-square test or Fisher exact test. Differences in DMR0 methylation ratio or IGF2 mRNA expression were assessed with the t-test. All statistical analyses were two-sided and P<0.05 was considered to be significant.

Results

Imprinting status of IGF2 in FAs and PTs. A total of 58 FAs and 27 PTs were subjected to Apa I polymorphism analysis and 25 FAs (43%) and 17 PTs (63%) were identified as heterozygous (A/g) for this polymorphism and used for the subsequent analysis. The frequency of LOI was higher for PTs (71%, 12/17) than for FAs (52%, 13/25) although the difference was not statistically significant. however, the imprinting status of IGF2 in FAs and PTs was not associated with age, tumor size, histological type or histological grade (Table I).

Relationship between LOI and IGF2 mRNA expression. IGF2 mRNA expression in 25 FAs and 17 PTs was determined by means of qRT-PCR and showed no significant difference between LOI positive (LOI+) and LOI negative (LOI-) tumors in either FAs or PTs (Fig. 3). The epithelial cells and stromal cells were then separately obtained from the six tumors (three FAs and three PTs) with the aid of LMD and subjected to the LOI assay. The IGF2 imprinting status of the epithelial cells and the stromal cells in each tumor was identical, i.e., three tumors contained LOI+ epithelial cells and LOI+ stromal cells and three tumors LOI- epithelial cells and LOI- stromal cells (Fig. 4A). On the contrary, IGF2 mRNA expression in most tumors (three FAs and two PTs) was upregulated in the stromal cells in comparison with that in the epithelial cells (Fig. 4B).

Table I. Imprinting status of IGF2 in fibroadenomas and phyllodes tumors.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>LOI (-)</th>
<th>LOI (+)</th>
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<th>P-value</th>
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<tr>
<td>Tumor histology</td>
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<tr>
<td>Age (years)a</td>
<td>22 (18-63)</td>
<td>30 (16-48)</td>
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<td>Tumor size (mm)a</td>
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<tr>
<td>Age (years)a</td>
<td>50 (21-52)</td>
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*aMedian (range).*

We also analyzed LOI and IGF2 mRNA expression in the normal tissues surrounding the tumors (6 FAs and 6 PTs). LOI was observed in four (33%) of the 12 normal tissues, while IGF2 mRNA expression was significantly lower in the normal tissues than in the tumors (P=0.041, Fig. 5).
Correlation between IGF2 mRNA expression and MED12 mutation. Of the 40 tumors (23 FAs and 17 PTs), 24 (12 FAs and 12 PTs) harbored MED12 mutations. No significant difference in IGF2 mRNA expression was observed between the tumors with and without MED12 mutations (Fig. 6A). In addition, MED12 mutation status showed no correlation with IGF2 imprinting status (Fig. 6B).

Relationship between LOI and IGF2 DMR0 methylation. The relationship between LOI and DMR0 methylation was examined in 14 FAs and 11 PTs which were polymorphic for both rs680 in exon 9 and rs3741210 in DMR0. There was no significant difference in DMR0 methylation ratios of either allele or a paternal allele between LOI+ and LOI- tumors (Fig. 7).

Discussion

In the present study, LOI was observed in 52% (13/25) of FAs and 71% (12/17) of PTs but the IGF2 mRNA expression was not associated with LOI, in contrast to the two classical studies on Wilms' tumors, which reported that IGF2 mRNA expression was two times higher in LOI+ than LOI- tumors (9,10). Although Kaneda et al reported that IGF2 was upregulated in LOI+ intestinal crypts in mice (27), no association of LOI with IGF2 mRNA upregulation has been reported by other investigators in studies of several types of tumors including breast cancer (7,17,28). We therefore considered it unlikely that LOI plays an important role in the upregulation of IGF2 mRNA in the majority of tumors including FAs and PTs.

We were able to confirm that IGF2 mRNA expression is significantly upregulated in FAs and PTs in comparison with surrounding normal tissues as previously described (7,21,29).
A separate analysis of IGF2 mRNA expression in the epithelial cells and stromal cells obtained by means of LMD demonstrated that IGF2 mRNA expression is more upregulated in the stromal than the epithelial cells. This observation seems to be consistent with the fact that, although FAs and PTs consist of both epithelial and stromal components, they essentially stem from overgrowth of the stromal cells since the stromal cells, but not the epithelial cells, harbor MED12 mutations (3,23,25). Thus, it is speculated that IGF2 plays a definite and significant role in the pathogenesis of these tumors even though the mechanism of its upregulation is not related to LOI.

Noteworthy, the imprinting status of the epithelial cells and stromal cells in each tumor was identical, i.e., every tumor consisted of either LOI+ epithelial cells and LOI+ stromal cells or LOI- epithelial cells and LOI- stromal cells. It has been reported that LOI can be induced by treatment with butyrate, which modifies histone acetylation (5). Furthermore, oxidative stress reportedly induces NF-κB binding to the CCCTC-binding factor (CTCF) promoter, which then leads to reduced CTCF expression, loss of CTCF binding to the ICR and IGF2 LOI (30). The epithelial cells of FAs and PTs often exhibit hyperplastic change, so that we

Figure 6. IGF2 mRNA expression and LOI in relation to MED12 mutation. Comparison of IGF2 mRNA expression levels (A) and frequency of LOI+ tumors (B) in MED12 mutant (n=24) and wild (n=16) tumors (fibroadenomas or phyllodes tumors). Black box, tumors with LOI; white box, tumors without LOI.

Figure 7. IGF2 imprinting status and DMR0 methylation. Comparison of DMR0 methylation ratios of both alleles for LOI fibroadenomas (A) and LOI+ phyllodes tumors (B), and of DMR0 methylation ratios of the paternal for LOI fibroadenomas (C) and LOI+ phyllodes tumors (D).
speculate that a certain factor produced from the stromal cells might stimulate the proliferation of the epithelial cells, and such a factor might also be implicated in the induction of LOI in the epithelial cells.

Consistent with previous observations regarding colon cancer, esophageal cancer and breast cancer that LOI was detected not only in tumor tissues but also in the matched normal tissues (28,31,32), we identified LOI in 33% of the normal tissues surrounding the FAs or PTs. It has been reported that the presence of LOI in normal tissues can be a risk factor for the development of colon cancer (33,34). Since LOI in the normal tissues from the healthy controls was not analyzed in the present study, it remains as yet unknown whether the presence of LOI in normal tissue represents a risk for the development of FAs or PTs.

Di Tommaso et al reported that leiomyomas with MED12 mutations expressed significantly higher levels of IGFB2 mRNA (26). However, we could not detect any significant association of MED12 mutations with the upregulation of IGFB2 mRNA levels, indicating that MED12 mutations perform a different role in the pathogenesis of leiomyomas and of FAs or PTs. DMR0 hypomethylation is reportedly associated with LOI in colorectal cancers (19) but not in other types of cancer (6,15). Since a significant association between LOI status and DMR0 methylation levels could not be demonstrated in our study, DMR0 methylation is unlikely to play an important role in the development of LOI in FAs and PTs.

In conclusion, we were able to demonstrate that IGFB2 mRNA expression is upregulated in the stromal cells in FAs and PTs, but that LOI of IGFB2 is not implicated in this upregulation. Furthermore, MED12 mutations were not found not to be associated with induction of LOI or IGFB2 mRNA upregulation, while DMR0 hypomethylation was found to be unlikely to play a significant role in the induction of LOI. Further studies are thus needed to clarify the role of LOI and the mechanism of IGFB2 mRNA upregulation in the pathogenesis of FAs and PTs.

References

3. Nordin M, Bergman D, Halje M, Engstrom W and Ward A: Frequent MED12 mutations with the upregulation of IGFB2 mRNA levels, indicating that MED12 mutations perform a different role in the pathogenesis of leiomyomas and of FAs or PTs. DMR0 hypomethylation is reportedly associated with LOI in colorectal cancers (19) but not in other types of cancer (6,15). Since a significant association between LOI status and DMR0 methylation levels could not be demonstrated in our study, DMR0 methylation is unlikely to play an important role in the development of LOI in FAs and PTs.

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