Wnt5a Cloning, Expression, and Up-Regulation in Human Primary Breast Cancers

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ABSTRACT

Wnt genes are involved in mouse mammary cancer, but their role in human cancer is unknown. Human Wnt5a was cloned from a placental cDNA library and used to assess expression by ribonuclease protection and in situ hybridization in human breast cell lines and in normal, benign, and malignant breast tissues. Human Wnt5a shows over 99% homology at amino acid level with mouse Wnt5a, and 90% with Xenopus Wnt5a. It was expressed only at low levels in breast cell lines and normal breast tissue. Benign proliferations and invasive cancer respectively showed 10-fold and 4-fold higher Wnt5a than normal breast tissues. The greater up-regulation in benign conditions suggests a role in aberrant differentiation. In situ hybridization localized the signal to the epithelial component. Wnt5a is the first member of the Wnt family to demonstrate overexpression in human breast cancer. It was not associated with factors known to affect breast cancer prognosis such as lymph node status or epidermal growth factor receptor status.

INTRODUCTION

In mouse mammary tumor virus-induced breast cancer, analysis of insertion sites has shown activation of endogenous genes, int genes (1). Int-1 (now Wnt1) was the first gene isolated and shows strong homology to a Drosophila developmental gene, wingless (2), involved in morphological development. The importance of their role is reflected by the severity of the phenotypic abnormalities that result from aberrant Wnt expression. Thus, Wnt genes have important roles in development and in cancer.

The role of Wnts in mouse mammary carcinogenesis has been extensively studied and there is evidence that they are secreted proteins, processed via the Golgi apparatus (6), which remain tightly associated with the extracellular plasma membrane or matrix (7). Wnts produce morphological effects on some mouse mammary cancer cell lines by transfection in autocrine (8) and paracrine mechanisms (9).

A survey of expression in normal mouse mammary gland development showed that some Wnts are expressed in virginal breast, some in pregnancy, and others in lactation (10). However, Wnt1, which is involved in carcinogenesis is not expressed in normal mouse mammary tissue. This implicates Wnt gene family members in normal breast development and suggests aberrant expression of other members can contribute to malignancies (11).

Evaluation of the normal expression of Wnt genes in human breast epithelium and cancer would contribute to understanding the role of Wnt genes in human cancer. It has been shown that some of the Wnt genes are expressed in human breast tissue, and that quantitative differences exist in the Wnt expression profile of normal and proliferative lesions (12).

We chose Wnt5a as a candidate human gene to clone and evaluate because it is expressed in normal mouse breast epithelium to a low extent, and also in mouse breast cancer cell lines (10). Furthermore it has some different properties from the human Wnt genes previously cloned in its effects on cell gap junctions (13) and Xenopus development (14). Interactions between Wnt genes with different normal functions may contribute to malignant transformation (11).

MATERIALS AND METHODS

Isolation of 384-Base Pairs Fragment of Human Wnt5a from Fetal Brain cDNA Library. Two hundred ng of a human fetal brain library in plasmid pCDMB (obtained from Dr. D. Simmons and Dr. J. Fawcett, Institute of Molecular Medicine, Oxford, United Kingdom) was used as a template for PCR. Amplification of cDNA was carried out using 500 ng of each of the degenerate forward (5’-GGGAATTCCTCA^CG^CTG^/ cAA^CG^TG^/CCAT-3’) and reverse (5’-AAATCTAGA^/ gCA^CGCACC^CG^TG^/cAA-3’) oligonucleotide primers previously described by Gavir et al. (5). PCR products were separated on a 2% agarose gel. Products of the correct size (predicted from known Wnt sequences) were recovered, then further amplified using the above primers and ligated into the plasmid pBluescript KS+ (Stratagene). JM109 cells were transformed with the reaction products, and the nucleotide sequences of clones containing inserts of correct size were determined by dideoxy chain termination sequencing. Clones with significant homology to known Wnts were identified using the FASTA program (CCG) and included one clone containing a 384-base pair fragment of human Wnt5a.

Isolation of Human Wnt5a from Placental cDNA Library. Replica colony lifts of approximately 10^7 recombinant clones of a human placental library in the plasmid pCDM8 were prepared using Hybond-N membranes (Amersham). The membranes were hybridized to a Wnt5a probe synthesized using the

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1 This work was funded by the Imperial Cancer Research Fund and Oxfordshire Health Authority.
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384-base pair fragment isolated as described above. The probe was generated by incorporating [α-32P]dCTP (Amersham) in a PCR amplification of the 384-base pair fragment using the above degenerate primers. The resultant species were end filled and separated from unincorporated nucleotides by passage through a Sephadex G-50 spin column (Boehringer Mannheim). After hybridization of the probe to the membranes, positive clones were identified by autoradiography, and subjected to further rounds of screening. After four rounds of screening, positive clones were selected and sequenced. Clones with significant homology to known Wnts were identified and included one containing 798 base pairs of the 3' end of the human Wnt5a cDNA as well as some 3' untranslated sequences.

Further sequence S' to the 798-base pair 3' terminus was obtained using a nested PCR strategy. A primary PCR reaction was carried out using 200 ng of a placental cDNA library in pCDM8 as template. The primers used were a mouse Wnt5a-specific forward primer F1 (5'-ATGAAGAAGCCCATTGG-GAATA-3') corresponding to the 21 extreme 5' nucleotides of mouse Wnt5a, and a human Wnt5a-specific reverse primer R1 (5'-GCACGCCGGCTATGGGTT-3') corresponding to a known sequence in the 798-base pair partial clone. Fragments of correct size (approximately 462 base pairs) were recovered and used as template for nested PCR. In the nested PCR reaction the forward and reverse primers (FP2 and RP2) were 3' and 5' of the primers F1 and R1, respectively. Primer FP2 (5'-AANTCNGTGTCNCCTNGG-3') was a fully degenerate primer, and primer RP2 (5'-GTGGTTATCCACAGTGCT-3') was a human Wnt5a-specific primer corresponding to the extreme 5' region of the 798-base pair partial clone. Fragments of correct size (approximately 234 base pairs) were recovered, subcloned into plasmid pBluescript SK, and sequenced. Clones with significant homology to Wnt5a were identified and included one containing 234 base pairs of human Wnt5a sequence S' to the 798-base pair partial clone.

A nested PCR strategy was also used in order to clone the extreme 5' region of Wnt5a. In a primary PCR reaction, 200 ng of human placental cDNA library in pCDM8 was used as template. The primers used were a pCDM8-specific primer and the human Wnt5a-specific internal primer R1. Products of a large enough size to contain the 5' end of Wnt5a were recovered and used as template in the nested PCR. In the nested PCR reaction, primers F1 and R2 were used. Fragments of correct size (approximately 366 base pairs) were recovered, cloned into TA cloning vector (Invitrogen), and sequenced. A clone containing the 5' end of Wnt5a was identified. Thus the sequences of the original 798-base pair partial clone and the two nested PCR products combined to give the full-length sequence.

Chromosomal Localization of Human Wnt5a. Twenty-µg DNA samples from a panel of human-rodent hybrid cell lines, and control human, mouse, and hamster DNA (obtained from Dr. N. Spurr, Imperial Cancer Research Fund, London, United Kingdom) were digested with EcoRI, fractionated on a 0.7% agarose gel, and transferred to Hybond-N membranes according to Southern's protocol (15). The membranes were then hybridized to a random primer generated [α-32P]dCTP-labeled probe, using a 1.4-kilobase XbaI fragment of the original (placental) partial human Wnt5a clone as template. Signals were detected by autoradiography.

Ribonuclease Protection Assays. Ribonuclease protection assays were carried as described in Ref. 16. Briefly, a 384-base pair fragment of human Wnt5a was cloned into pBluescript KS. The EcoRV linearized plasmid was used to generate antisense [α-32P]CTP-labeled probes with T7 RNA polymerase (Gibco). Probes were hybridized to 10-µg samples of total RNA extracted from tissues and cell lines by a single-step extraction method (17). Hybridization was carried out at 45°C for 16 h. Each hybridization also contained an antisense probe for GAPDH as a loading control. GAPDH probes were prepared from a 120-base pair b fragment of GAPDH cloned into pBluescript SK (18). Unhybridized probe was digested with RNase A and T1, and protected fragments were electrophoresed on polyacrylamide gels. Dried gels were autoradiographed.

Image Analysis. Autoradiographs of ribonuclease protection assays were scanned using a Bio-image analyser (Milligan Bioresearch) to determine RNA abundance. Wnt5a values were normalized to GAPDH to allow for loading. MCF10A RNA was included in all assays as a positive control. This was assigned a unit level of expression and all other values were standardized to this.

Cell Lines. The following breast cell lines were obtained from ATCC (Bethesda, MD): T47D (ATCC HTB133), MDA231 (ATCC HTB26), MCF10 (ATCC CRL10317), MDA415 (ATCC HTB128), MDA453 (ATCC HTB131), MDA157 (ATCC HTB24), BT20 (ATCC HTB19), SKBR3 (ATCC HTB30). ZR9B11 (ZR-75-1), ZR4, and ZR11 were obtained from Dr. E. Valvenius (Department of Pathology, University Hospital, Uppsala, Sweden). MCF7s were obtained from Dr. B. Durkacz (Cancer Research Unit, University of Newcastle upon Tyne). Adriamycin-resistant MCF7s were obtained from Dr. K. Cowen (National Institutes of Health). Lines 2-5-2a, 3-4-1, 5-3-1, 6-1-1, MTSV1-7, and MTSV4-1 were obtained from Dr. J. Taylor (Imperial Cancer Research Fund, London, United Kingdom).

Cell Culture. T47D, MDA231, MCF10, MDA453, SKBR3, MCF7, Adriamycin-resistant MCF7, ZR9B11, ZR4, and ZR11 were maintained in DMEM with 10% FCS. BT20 was maintained in Eagle's MEM supplemented with 15% FCS and 2 mm glutamine. MDA415 was maintained in DMEM, 15% FCS, 1 µm hydrocortisone, 10 µg/ml insulin, and 10 µg/ml glucocorticone. MDA157 was maintained in RPMI 1640 and 10% FCS. MTSV1-7, MTSV4-1, 2-5-2a, 3-4-1, 5-3-1, 6-1-1 were maintained in DMEM: Ham's F12 (1:1), 10% FCS, 10 µg/ml insulin, and 5 µg/ml hydrocortisone. All cultures were grown on plastic dishes in 5% CO2-95% air in humidified incubators. All cultures were free of Mycoplasma.

Handling of Clinical Samples. Protocols for handling of clinical samples and assays for hormone and growth factor receptors were followed as detailed in LeJeune et al. (19). Briefly, Tumors were considered to be ER positive if they contained at least 10 fmol of specific binding sites per mg of cytosolic protein, and EGFR positive if they contained at least

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The abbreviations used are: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ER, estrogen receptor; EGFR, epidermal growth factor receptor; ATCC, American Type Culture Collection.
20 fmol of specific binding sites per mg of membrane protein. Human tissue samples were selected to represent normal breast tissue, benign breast disease, and breast cancer.

**In Situ Hybridization.** A single-stranded antisense RNA probe to Wnt5a was transcribed from EcoRI-linearized Wnt5a DNA using T3 DNA polymerase (Promega), and 32P-UTP (~800 Ci/mmol; Amersham International) as the sole source of UTP. Histological sections of human tissues that had been fixed in neutral-buffered formalin and embedded in paraffin wax were treated in the manner described by Senior et al. (20) with minor modifications. In summary, 1 × 10^6 cpm of unhybridized probe in 10 ml buffer was hybridized overnight at 55°C to sections permeabilized with proteinase K. Posthybridization steps included several large volume washes in a 50% formamide buffer at 55°C to remove unhybridized probe. RNase A treatment to digest single-stranded and imperfectly hybridized domains, and extensive washing to remove these cleaved fragments were performed. The final washes were in 0.5X SSC (1X SSC is 0.15 M NaCl and 0.015 M sodium citrate) at 65°C for 30 min twice. Slides were dehydrated and processed for autoradiography (Ilford K2) at 4°C for 7 to 10 days. Latent images were developed with Kodak D-19, and sections were Giemsa counterstained.

The β-actin mRNA was used as a positive control for the presence of mRNA species, as described previously by Wright et al. (21). Labeled Wnt5a sense transcripts were used as controls for nonspecific riboprobe binding.

**RESULTS**

Isolation of Wnt5a cDNA. A partial length cDNA was initially isolated by using PCR primers to homologous domains in Wnt genes. This was used to isolate a cDNA from a human placental library, which, in combination with nested PCR products from the same library provided the full human Wnt5a cDNA coding sequence shown in Fig. 1 with the corresponding amino acid sequence. The sequence comparison of human Wnt5a with mouse and Xenopus Wnt5a shows extensive conservation with amino acid level homology of 99% and 90%, respectively.

Chromosomal Localization of Wnt5a. The chromosomal location of the human Wnt5a was determined by screening hamster-human and mouse-human hybrid cell lines which had known human chromosome karyotypes. Southern blotting showed a Wnt5a signal located on human chromosome 3 (Fig. 2), which was absent in a mouse-human hybrid cell line carrying a p11 to p terminus deleted chromosome 3 (data not shown). This suggests that Wnt5a is on chromosome 3p.

Expression of Wnt5a in Human Breast Cell Lines. To assess expression in human breast cancer, a panel of human breast cell lines was initially analyzed, since they represent a pure epithelial population. Cell lines from normal breast duct luminal epithelium, benign epithelial proliferation, and in situ or invasive components of breast epithelium were studied (Table 1 and Fig. 3). Levels of expression measured by nuclease protection assays were low, requiring 7 days of development. One line from luminal epithelium had higher levels (MTSV1-7) than the others.

Cell lines established from malignant pleural effusions and metastasis had lower levels than the luminal cell line MTSV1-7 in all but one case (BT20). Thus, in vitro cell lines rarely expressed Wnt5a.

Expression in Human Breast Tissue. Expression was then studied in normal breast tissue, tissue from benign breast diseases, and primary breast cancers. The clinical details of the patients (e.g., age, tumor receptor status) are given in Table 2.
Wnt5a Up-Regulation in Human Breast Cancer

suggests that F', i.e., chromosome 20 and partial 4; r, chromosome 2; hamster genomic DNA. 3, Fig. 2 Southern blot of human-rodent hybrid cell lines with human

Table I Comparison of Wnt5a expression in breast cell lines derived from ductal epithelium, in situ and invasive components of breast carcinoma, and breast cancer metastases

Normal tissues (n = 15) were obtained either from reduction mammoplasties (n = 7), or from normal tissue adjacent to tumors (n = 8). Benign breast disease samples consisted of fibroadenomas (n = 5), fibrocystic disease (n = 3), and benign phyllode tumors (n = 2). Tumors (n = 28) were selected to represent different subgroups according to known prognostic factors (node, ER, and EGFR status). In contrast to the cancer cell lines, Wnt5a was commonly expressed in primary breast cancer (Fig. 4). Comparing normal breast tissue to tumor tissue showed levels that were 4-fold higher on average in the latter (Table 2). Ten of 28 tumors had levels higher than the highest expression in the cell lines from normal breast tissue. Thus, the cell lines reflected normal breast expression, but not tumor levels. Tumor levels were significantly greater than normal tissue levels (P = 0.0004, Mann-Whitney U test).

Benign breast tissue also showed much higher expression than normal breast tissues, similar to and often greater than levels in many of the tumors. Two different types of benign breast disease were studied: fibroadenomas and fibrocystic disease. The former is a benign lesion involving epithelial and stromal elements. The latter is a nontumorous collection of cysts and ducts with some epithelial hyperplasia. In both types of benign breast disease there was high expression of Wnt5a (Fig. 4, nuclease protection). Benign levels were significantly greater than those of normals (P = 0.0012 Mann-Whitney U test) and also than those of tumors, although to a smaller extent (P = 0.03).

In situ hybridization using the Wnt5a probe was carried out to assess localization of expression. This showed that levels were low and difficult to detect in normal breast but were detectable in a few ducts and lobules. In some larger ducts the mRNA appeared to be expressed in luminal cells but not the myoepithelial population. In fibroadenomas there was high expression in the epithelial component uniformly throughout the tumor (Fig. 5, a and b). In fibrocystic disease it was again...
was not due to gene amplification since Southern blots of the breast cancers. Wnt5a probes used as controls for nonspecific hybridization showed a uniform and low background signal (data not shown).

The sense clumps of invading cells throughout the stroma. There was no expressed within the epithelial element of the tumor and in breast cancer cell lines. RNase protection assay on cell lines. Fig. 3 a, b, 3-4-1; c, 5-3-1; d, 6-1-1; e, MT5V1-7; f, MRSV4-1; g, MCF10; h, ZR11; i, ZR4; j, MDA415; k, MDA453; l, MDA457; m, BT20; n, SKBR3; o, Adriamycin-resistant; p, MCF7; q, AR9B11; r, T47D; s, MDA231.

![Fig. 3 RNase protection assay on cell lines. Wnt5a and GAPDH signals are shown. Lanes a–f, human mammary epithelial cell lines; Lanes g–s, human breast cancer cell lines. a, 2-5-2a; b, 3-4-1; c, 5-3-1; d, 6-1-1; e, MT5V1-7; f, MRSV4-1; g, MCF10; h, ZR11; i, ZR4; j, MDA415; k, MDA453; l, MDA457; m, BT20; n, SKBR3; o, Adriamycin-resistant; p, MCF7; q, AR9B11; r, T47D; s, MDA231.](image)

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* Min, minimum; max, maximum.

Table 2 Wnt5a expression assayed by RNase protection in human breast cancer

expressed in the epithelial component (Fig. 5, c and d). Similarly, in the malignant tumors (Fig. 5, e and f) Wnt5a was expressed within the epithelial element of the tumor and in clumps of invading cells throughout the stroma. There was no increased localization at the invading edge. The sense Wnt5a probes used as controls for nonspecific hybridization showed a uniform and low background signal (data not shown).

Thus, in many cases of benign breast proliferation and breast cancers. Wnt5a is overexpressed in the epithelium. This was not due to gene amplification since Southern blots of the high-expressing cases showed no evidence of this (data not shown).

Features of carcinomas known to relate to tumor phenotype were compared with Wnt5a expression. There was no correlation of either ER or EGFR with Wnt5a expression (P = 0.3 and 0.5, respectively, Spearman’s rank correlation coefficient; Table 2), nor was there a correlation with node status (P = 0.1). There was no association with menopausal status, as indicated by age >50 years or <50 years old (Table 2). ER and EGFR were inversely related to each other, as previously reported (Ref. 22; P = 0.005, Spearman’s rank correlation coefficient). ER was related to age and EGFR inversely as has been described before (Ref. 22; P = 0.0007 and 0.036, respectively, Spearman’s rank correlation coefficient).

In relation to patient age, there was no significant difference between normal and benign samples but the primary cancer patients were significantly older than the normal (P = 0.02) and benign (P = 0.0013) samples. However the differences in Wnt5a expression between the samples cannot be a function of the different age distributions, as Spearman’s rank correlation of Wnt5a with age as a continuous variable shows no correlation (P = 0.11). Furthermore the group of tumors which overlap the normal and benign samples in age (n = 9, age <52 years) have no different a level of Wnt5a than those which are older (n = 19, age >52 years, P = 0.86). Also, the differences in the level of Wnt5a between normal samples and cancers are maintained whether the cancer patients considered are over or under 52 years of age. Thus, the significant differences in Wnt5a expression seen between the groups are not related to age distribution differences.

**DISCUSSION**

The human Wnt5a gene shows marked homology to other species including mouse (23) and Xenopus (24). This is characteristic of all of the Wnt family members which are highly conserved (25). They show greater homology to the family member in different species than to other family members expressed in the same species. Wnt5a is located on the terminal region of chromosome 3 beyond the 3p11 band, whereas mouse Wnt5a is on chromosome 14. However, human chromosome 3 is syntenic with mouse chromosome 9, suggesting chromosomal rearrangements at this locus during evolution. The region 3p21-25 is known to be involved in loss of heterozygosity in human cancer (26), including 30% of breast cancers (27). How-
ever, the work of Clark et al. (28) suggests that Wnt5a is outside this region and therefore not the gene involved.

During the course of this work, the isolation of overlapping clones was reported giving the total sequence of the human Wnt5a cDNA isolated from a human fetal fibroblast library. We have similarly cloned overlapping clones from a human placental library. Our results independently confirm the sequence published by Clark et al. (28), as well as the chromosomal localization of the gene. Our sequence differs from that of Clark et al. (28) at a few nucleotides but these do not give rise to amino acid differences.

Expression was studied in a range of human breast cancer cell lines representing ER-positive and -negative types as well as those having amplification of EGFR or erbB-2. With the exception of BT20, expression in the cell lines was very low with levels similar to or lower than those found in normal tissues. The breast cancer cell line BT20 showed high wnt5a expression, similar to the elevated levels found in benign proliferative lesions. In the mouse, Wnt1 expression in mammary epithelium produces abnormal morphology in a hormone-independent fashion (29). In relation to this, the level of expression of Wnt5a in breast cancer cell lines appears unrelated to hormone receptor status.

In tissues, Wnt5a expression was generally higher than that in cell lines. Benign and malignant proliferative lesions of the breast respectively showed levels of Wnt5a 10-fold and 4-fold
higher than those in normal tissue. In the mouse Wnt5a is expressed in normal breast tissue and its regulation has been studied over a short period of reproductive history (10). Wnt5a is present in early pregnancy but is undetectable by day 17.5 of pregnancy. It is clearly difficult to reproduce such studies in humans, but our results suggest that human breast tissue generally resembles that of the mouse in its expression of Wnt5a, although no major endocrine effects involving steroid hormones were demonstrated in that pre- and postmenopausal breast levels were no different in any of the patient groups. This does not, however, exclude pregnancy-specific regulation events. Wnt5a was highest in the benign proliferative lesions, and analysis in human breast cancer showed up-regulation of Wnt5a in 10 of 28 carcinomas above the highest level in normal breast tissues. We have reported that the level of RNA must be in the fibroadenoma epithelium and a smaller decrease in the expression of Wnt4 and a smaller decrease in the expression of Wnt5a. Thus, it is possible that Wnt5a itself may interfere with the function of other Wnts in human breast tissue where it is overexpressed.

To assess localization of Wnt5a, in situ hybridization was carried out on normal tissues, fibrocystic disease, fibroadenomas, and carcinomas. In normal tissues, the level of expression was generally too low to detect above background, but could be seen in a few ducts. In fibrocystic disease, fibroadenomas, and carcinomas Wnt5a was expressed in epithelium. In some cases it was possible to clearly resolve the basal myoepithelial layer of the breast from the luminal epithelium and Wnt5a was expressed in the luminal cell layer.

In fibroadenomas stromal cells are present in a much greater proportion than in normal epithelium. Dilution of RNA from the epithelium by matrix and stromal cells shows how high the level of RNA must be in the fibroadenoma epithelium compared to normal tissue epithelium. It is possible that the stromal cells are involved in regulation of Wnt5a expression in the epithelium or conversely that Wnt5a expression effects stromal growth. Coculture of luminal cells with stromal cells to measure Wnt5a regulation would be helpful to assess the role of such stromal epithelial interactions.

The up-regulation of Wnt5a in both benign and malignant proliferative disease of the breast suggests an important role of Wnt genes in breast pathology. Wnt5a is the first member of the family to demonstrate up-regulation in human breast cancer. Cell lines established from benign or normal tissues may be suitable models for further evaluation of the role of Wnt5a to assess its regulation under variable growth and differentiation conditions.

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