Differentially Regulated Genes as Putative Targets of Amplifications at 20q in Ovarian Cancers

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Frequent amplification of DNA at 20q or part of 20q has been demonstrated by comparative genomic hybridization in ovarian cancer (OC), but the genetic target(s) of these amplifications remain unknown. We examined copy-number changes with respect to six candidate genes, E2F1 (20q11.2), TGIF2 (20q11.2), AIB1 (20q12), PTPN1 (20q13.1), ZNF217 (20q13.2), and BTAK (20q13.13), and then measured transcription levels of each candidate in 18 OC cell lines. Three distinct cores of amplification were identified: 20q11.2, harboring E2F1 and TGIF2 (region I; 1 of 18 cell lines, 5.6%); 20q13.1, harboring PTPN1 (region II; 5 lines, 27.8%); and 20q13.2, harboring ZNF217 and BTAK (region III; 6 lines, 33.3%). Among the six genes examined, expression levels of PTPN1 and ZNF217 were significantly correlated with absolute copy-number, and those of PTPN1 and TGIF2 were significantly correlated with copy-number relative to the centromere of chromosome 20 (2cen). Among 19 primary OCs examined, moreover, we observed amplification of TGIF2, PTPN1 and ZNF217 in five (26.3%), ten (52.6%), and twelve (63.2%) tumors, respectively. Expression levels of PTPN1 and ZNF217 were significantly correlated with their copy-numbers in those primary OCs. Our results suggest that 20q amplifications in OCs can be extensive and complex, probably due to synergistic or non-synergistic amplification of separate regions of 20q, involving multiple, independently amplified targets.

Key words: 20q — Amplification — Ovarian cancer

Among a variety of genetic alterations that may occur during development and progression of ovarian cancer (OC), DNA amplification is of particular importance because it can activate specific genes that confer a selective growth advantage. The regions most frequently amplified in OC, 17q21, 8q24 and 12p11, contain the ERBB2, MYC, and KRAS genes, respectively. Recently, additional but less fully characterized amplicons have been identified by means of comparative genomic hybridization (CGH). Among them, 20q or part of 20q appears to be amplified in 20–50% of OCs. This region is often amplified in other types of tumor as well, including breast, colon, and pancreatic cancers, where amplification of 20q has been implicated in metastasis and poor prognosis. It is reasonable to suppose that 20q may harbor dominantly acting genetic elements that contribute to the progression of OC, as well as other types of tumors.

Levels and patterns of amplification at 20q appear to be highly various and complex. Three distinct non-syntenic regions, 20q11, 20q12-13.1, and 20q13.2, have been identified in breast cancers. Using array-CGH, Pinkel et al. also detected complex changes in copy-number and structure of the 20q region in breast cancers; that is, 20q seems to harbor more than two genes as targets for amplification. Although on functional grounds several candidates in the region, e.g. E2F1 (20q11.2), TGIF2 (20q11.2), AIB1 (20q12), PTPN1 (20q13.1), ZNF217 (20q13.2), and BTAK (20q13.2), have been proposed as targets for the 20q amplification in breast cancer, targets in OC remain unknown. In order to reveal some of these targets, we examined the six genes in OC cell lines and primary tumors. To be defined as a target of an amplification event, a gene should be over-expressed and functionally activated via an amplification mechanism. Therefore we compared the copy-number status of each of the six candidates with their expression status in our OC materials.

MATERIALS AND METHODS

Cell lines and primary tumors Among the total of 18 OC cell lines examined, information about 14 of them
(HMOA, HUOA, HKOA, HTOA, HIOA, W3UF, HTBOA, MCAS, Kuramochi, TYK-nu, TYK-nu.CP-r, RMG-I, RMG-II, and RMUG-L) has been published. The other four lines (OVCA3, OVCAR5, OVCAR8, and SKOV3) were purchased from American Type Culture Collection (ATCC). All lines were maintained in RPMI-1640 with 10% fetal calf serum and penicillin/streptomycin.

Nineteen unselected primary ovarian tumors (7 serous adenocarcinomas, 6 clear-cell carcinomas, 4 endometrioid adenocarcinomas, and 2 mucinous adenocarcinoma) were obtained from the Department of Obstetrics and Gynecology, National Nagoya Hospital or from Fukushima Medical University, with written consent from each patient in the formal style and after approval by the local ethics committee. All tumors were histologically classified with hematoxylin and eosin staining by one gynecological pathologist, and contained over 60% tumor cells.

**Fluorescence in situ hybridization (FISH)** Metaphase chromosomes slides were prepared from cell lines or normal peripheral blood lymphocytes for FISH experiments that were carried out in a standard manner.

FISH analyses were performed using as probes biotin-16-dUTP- or digoxigenin-11-dUTP-labelled bacterial artificial chromosomes (BACs) or P1 artificial chromosomes (PACs) containing the six candidate genes, as follows: E2F1 (RP11-607H21), TGIF2 (RP4-277B1), AIB1 (RP11-19D5), PTPN1 (CITB-2022M04), ZNF217 (RP4-724E16), and BTAK (GS-491G11). A PAC RP5-1059A9 specific for the centromere of chromosome 20 (20cen) was used as a reference.

Chromosomal in situ suppression hybridization and fluorescent detection of hybridization signals were carried out as described elsewhere. The copy-number and molecular organization of the region of interest were assessed according to the hybridization patterns observed on both metaphase and interphase chromosomes. The copy-number of each locus was expressed as the absolute number of signals, or as the number of signals relative to the number of 20cen signals. Precise localization of each BAC was confirmed by FISH using normal metaphase chromosomes.

**Northern blotting** Ten-microgram aliquots of total RNA extracted from each cell line were electrophoresed in 1.0% agarose/0.67 M formaldehyde gel, and then transferred to a positively charged nylon membrane (Hybond N*, Amer sham Pharmacia Biotech, Tokyo). Probes for northern blotting consisted of Integrated Molecular Analysis of Genome and their Expression (IMAGE) cDNA clones 236142 (E2F1), 502333 (AIB1), 1560118 (PTPN1), 1360920 (ZNF217), and 1137958 (BTAK) purchased from Incyte Genomic, Inc. (St. Louis, MO); in addition, we used a full-length cDNA clone of TGIF2 (18) and a control probe (glyceraldehyde-3-phosphate dehydrogenase, GAPDH). All probes were labeled with [α-32P]dCTP and hybridized to pre-hybridized blots. The hybridized blots were washed and exposed as described elsewhere. Auto- radiographic signals were analyzed using Mac Bas (Fujifilm, Tokyo); each signal was normalized by reference to the value obtained for GAPDH and reported as a normalized expression level.

**Real-time quantitative polymerase chain reaction (PCR) and reverse transcriptase-PCR (RT-PCR)** The quantification of genomic DNA and messenger RNA (mRNA) levels of genes in primary tumors was carried out using a real-time fluorescence detection method. Single-stranded complementary DNA (cDNA) was generated from total RNA using “SuperScript” First-Strand Synthesis System (Invitrogen, Carlsbad, CA) following the manufacturer’s directions. Real-time quantitative PCR was performed using a LightCycler (Roche Diagnostics, Tokyo) with CYBR Green according to the manufacturer’s protocol. β2-Microglobulin (B2M) and GAPDH were used as the endogenous controls for genomic and mRNA levels, respectively, and genomic DNA copy-number and expression level of each sample were normalized on the basis of B2M and GAPDH levels, respectively. Primer sequences for each gene are available on request. Duplicate PCR amplification was performed for each sample.

**Statistical analysis** The relationship between copy-number or relative copy-number and the normalized expression level or relative copy-number of each gene was calculated using Spearman’s test, and correlation coefficients and associated probability (P) were calculated. P<0.05 was required for significance.

**RESULTS**

**Amplification status at 20q in OC cell lines** We first evaluated the copy-number status of the six positional and functional candidate genes by FISH in 18 OC cell lines (Fig. 1). CGH analysis had been carried out previously in 14 of these lines, and gain of DNA on 20q had been detected in eight of them (57%). By FISH, however, copy-number increases in 20q or part of 20q were detected in all lines. As shown in Figs. 1 and 2, four patterns of DNA increase along 20q were found: group A, gain of chromosome 20; group B, gain of the whole q-arm of chromosome 20 (whole-20q gain); group C, gain of specific loci (fewer than 5 of the 6 loci) without whole-20q gain; and group D, gain of specific loci with whole-20q gain. We defined as “cores of amplification” regions containing the loci with increased copy-numbers in groups C and D (Fig. 1). When the prevalence of the core of amplification was evaluated, we found three distinct regions along 20q, i.e. 20q11, harboring E2F1 and TGIF2 (region I; 1 of 18 cell lines, 5.6%); 20q13, harboring PTPN1 (region II; 5/18, 27.8%); and 20q13.2, harboring ZNF217 and BTAK (region III; 6/18, 33.3%) (Fig. 1). The cores of amplification in cell lines RMG-I and RMG-II (2/
18, 11.1%) spanned region II and region III. Among the eight cell lines of groups A and B (44.4%) we detected no cores of amplification (Fig. 1), although gain of the whole q-arm had been observed in cell lines of group B.

**Comparison of DNA copy-numbers or relative copy-numbers with expression levels of candidate genes in OC cell lines**

To determine whether amplification of candidate targets in 20q was consistently associated with their expression levels, we examined transcription of the six genes in question. Fig. 2 shows images from northern-blot and FISH analyses of ZNF217 and PTPN1 in representative cell lines. Levels of ZNF217 expression in cell lines with increased copy-numbers (HTOA and HUOA) were higher than in cell lines with low copy-numbers (HMOA and RMG1). Results in the same cell lines were similar for PTPN1 (data not shown). We were able to validate a significant and positive correlation between expression level and copy-number only for PTPN1 and ZNF217, indicating that those two genes are consistently over-expressed in some OCs in a gene dosage-dependent manner (Fig. 3A). Non-significant but weak correlations were observed for AIB1 (P=0.057) and TGIF2 (P=0.069), but the copy-number of BTAK did not correlate with its expression level at all (P=0.676).

On the other hand, levels of PTPN1 expression in cell lines showing relative increases in copy-number (Kuramochi and OVCAR5) were much higher than in cell lines without relative increases (HMOA and W3UF; Fig. 2B). We had observed the same result with TGIF2 in earlier experiments.18 We observed significant and positive correlation between expression levels and relative copy-numbers only for PTPN1 and TGIF2; thus, these two genes were consistently over-expressed through an increase in DNA relative to 20cen (Fig. 3B). No significant correlation between these parameters was observed for the other four genes.

**Amplification and expression status of potential target genes in primary tumors of OC**

Since TGIF2, PTPN1,
and ZNF217 were present in the “core of amplification” and their expression levels correlated with either the absolute copy-number or the copy-number relative to 20cen, or both, we analyzed copy-number and expression status of these three genes in 19 primary OCs. Since limited amounts of DNA and RNA were available from primary tumors, we determined copy-number and expression level of each gene using a real-time quantitative PCR.25–27) As shown in Fig. 4A, clear amplifications of TGIF2, PTPN1, and ZNF217 were observed in five (26.3%), ten (52.6%), and twelve (63.2%) tumors, respectively, if amplification was defined as the ratio of each gene/B2M greater than 2. Interestingly, a statistically significant correlation was observed between copy-number of PTPN1 and ZNF217, suggesting that co-amplification of these two genes may frequently occur in primary OCs (Fig. 4B). In addition, relative expression levels of PTPN1 and ZNF217 were significantly correlated with their copy-numbers normalized on the basis of B2M (P=0.0174 and 0.0087, respectively; Fig. 4C), while the relative expression level of TGIF2 was of marginal significance (P=0.0683; data not shown).

**DISCUSSION**

Although amplification at 20q or part of 20q has been demonstrated by CGH in a number of ovarian tumors,2–5) the target genes for the amplification event(s) remain unknown. As in other types of tumors such as breast cancer, gain or amplification at 20q appears variable in OC and many genes that could be targets of amplification events are present in the region. In the present study, therefore, we first examined the change in copy-numbers of six genes that emerged as likely candidates from a survey of the literature covering a relatively large series of OC cell lines, and we identified three distinct cores of amplification. However, since genomic analysis alone did not enable us to evaluate the roles of these genes in the tumorigenesis of OC, we measured transcription levels of these candidates in 18 OC cell lines and a panel of primary tumors. A similar strategy has guided studies to explore target genes for relatively large amplified regions, such as 17q22-23 in breast cancer28–31) or 3q26 in several other types of cancer.21, 32–34)

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**Fig. 2.** (A) Upper: northern-blot analysis of ZNF217 in four representative OC cell lines. Middle: DNA copy-number at the ZNF217 locus as determined by FISH (see Fig. 1). Lower: representative FISH image from the ZNF217-specific probe in the HTOA cell line. The two cell lines (HTOA and HUOA) having higher copy-numbers of ZNF217 clearly showed higher expression of this gene than two other cell lines (HMKOA and RMG1) having lower copy-numbers. (B) Upper: representative result of northern-blot analysis of PTPN1 in four different OC cell lines. Middle: relative DNA copy-numbers (/20p) at the PTPN1 locus determined by FISH analysis. Lower: representative image of two-color FISH analysis using a PTPN1-specific probe (red) and a 20cen probe (green) in the Kuramochi cell line. Cell lines Kuramochi and OVCAR5, with higher relative copy-numbers of PTPN1 clearly showed higher expression of this gene than two cell lines (HMOA and W3UF) with lower relative copy-numbers.
Although amplified chromosomal regions usually span a region of several Mb, which consists of several amplification units and harbors many different genes, the number of target genes activated via amplification is usually limited.\(^{22, 27, 35, 36}\) Despite amplification, several genes in the amplicon are not up-regulated, even though they have previously been nominated as candidate targets for amplification or are known to be oncogenes in specific types of tumors,\(^{35, 36}\) and the reasons for that have not been elucidated yet. Two criteria are necessary for defining an amplification target in tumors of interest: (a) the putative target gene is within the core of the amplification, and (b) amplification consistently leads to over-expression of the gene.\(^{17, 21, 22}\) Using these criteria we attempted to determine possible targets within the 20q amplicon in OCs, and identified significant correlations between expression levels and absolute or relative copy-numbers of TGIF2, PTPN1, and ZNF217. Each of these three genes was located in a separate core of amplification. Their amplification and increased expression dependent on DNA copy-number were also observed in primary OC tumors, although the relation between copy-number and expression level of TGIF2 was of marginal significance.

Among the six genes examined, expression levels of PTPN1 and ZNF217 were significantly correlated with absolute copy-number, whereas those of PTPN1 and TGIF2 were significantly correlated with copy-numbers relative to 20cen. Amplification and concomitant increases in expression of candidate genes are strong evidence for identifying targets for amplification that may be associated with development and/or progression of tumors. It remains unclear whether any biological differences exist between the increases in absolute as opposed to relative copy-numbers. However, increases in relative copy-number of 20q13.2 in colorectal cancer, and of MYC in prostate cancer, have been correlated with metastatic potential and poor survival outcomes, respectively.\(^{37, 38}\) Those findings suggest that not only numerical aberrations of DNA segments, but also a change in copy-number of specific regions on chromosomes, are associated with some tumor phenotypes. Genes undergoing gains in copy-number might be structurally or functionally affected by nearby chromosomal regions, or even by regions distant from them.

ZNF217 encodes a Krüppel-like zinc-finger transcription factor; this gene was originally identified based on its core location in the 20q13.2-amplicon observed in some breast cancers.\(^{17}\) In colorectal cancers, amplification of ZNF217 has been associated with increased metastatic potential.\(^{38}\) Constitutively aberrant expression of ZNF217 in human mammary epithelial cells promotes immortalization by overcoming senescence, increasing telomerase activity, and resisting inhibition of growth by transforming growth factor-beta (TGFβ).\(^{39}\) In the present study we demonstrated that ZNF217 is present in the region of 20q most frequently amplified among ovarian-cancer cell lines (region III, Fig. 1), and was concomitantly over-expressed in cells containing the relevant amplicon. All of these data together suggest that ZNF217 may be a strong candidate for oncogenesis in 20q-amplified OCs.

We recently identified TGIF2 as a novel TALE-superclass homeobox gene and a possible target for 20q-amplification in OC cell lines.\(^{40}\) According to Melhuish \textit{et al.},\(^{40}\) TGIF2, like TGIF, interacts with Smad3 to repress TGFβ-responsive transcription. We suggest that increased expres-

![Fig. 3. Correlation between copy-number (A) or relative copy-number (B) and expression level of each gene in 18 OC cell lines. Copy-numbers (x axes) were obtained from FISH analyses; relative copy-numbers were calculated by dividing the copy-number of each gene by that of the 20cen reference (see Fig. 1). Normalized expression levels (y axes) were calculated from the intensity of each signal on a northern blot, divided by the corresponding reference value obtained from GAPDH expression (see ref. 21). Significant results (P < 0.05) are shown in boldface type. CC: correlation coefficient.](#)
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Fig. 4. Amplification and expression status of TGIF2, PTPN1, and ZNF217 in 19 primary OCs. (A) Genomic copy-number ratios (upper) and relative expression levels (lower) of TGIF2, PTPN1, and ZNF217 normalized on the basis of B2M and GAPDH, respectively. Asterisks indicate the cases showing the clear increase in copy-number (>2.0 relative to B2M) of each gene. □ TGIF2, □ PTPN1, □ ZNF217. (B) A statistically significant correlation was observed between copy-number of PTPN1 and ZNF217 relative to B2M, suggesting that co-amplification of these two genes may occur frequently in primary OCs. (C) In 19 primary OCs, a statistically significant correlation between copy-number and relative expression level of candidate gene normalized on the basis of B2M and GAPDH, respectively, was observed in PTPN1 and ZNF217. Significant results (P<0.05) are shown in boldface type. CC: correlation coefficient.

Amplification of TGIF2 may play a role in tumor progression by decreasing the ability of TGFβ signals to control transcription of cell-cycle regulators.

PTPN1, encoding a non-receptor tyrosine phosphatase, is often over-expressed in breast and ovarian cancers. In contrast to our results, Tanner et al. found no correlation between amplification and expression of PTPN1 in breast cancers. It is possible that expression of PTPN1 might depend more strictly on its copy-number in ovarian tissues than in breast tissues.

We observed no significant correlation between expression level and absolute or relative copy-number for two outstanding candidates, AIB1 and BTAK. AIB1, encoding a member of the SRC-1 family of nuclear-receptor coactivators, is amplified and over-expressed in many types of tumors including breast and ovarian cancers. AIB1 interacts with estrogen receptors in a ligand-dependent manner, enhancing estrogen-dependent transcription. The fact that over-expression of AIB1 does not always coincide with its amplification in breast and gastric cancers is consistent with our results. BTAK, encoding a centrosome-associated kinase, is involved in abnormalities of centrosome duplication-distribution in mammalian cells; up-regulation of this gene leads to amplification of centrosomes, resulting in chromosomal instability and aneuploidy. Amplification and over-expression of BTAK have been detected in several cancer types, but its over-expression was not related to amplification in those experiments. Expression of AIB1 and BTAK, therefore, is likely to be regulated by mechanisms other than amplification, such as activation of transcriptional machinery.

Amplifications of 20q in OCs appear to be extensive and complex, probably as a result of synergistic or non-synergistic amplifications of separate regions involving
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