

Notch3 Gene Amplification in Ovarian Cancer

Joon T. Park,^{1,2} Mei Li,¹ Kentaro Nakayama,² Tsui-Lien Mao,² Ben Davidson,³ Zhen Zhang,² Robert J. Kurman,^{1,2} Charles G. Eberhart,² Ie-Ming Shih,^{1,2} and Tian-Li Wang¹

Departments of ¹Gynecology and Obstetrics and Oncology, and ²Pathology, The Johns Hopkins Medical Institutions, Baltimore, Maryland; and ³Department of Pathology, Norwegian Radium Hospital, University of Oslo, Oslo, Norway

Abstract

Gene amplification is one of the common mechanisms that activate oncogenes. In this study, we used single nucleotide polymorphism array to analyze genome-wide DNA copy number alterations in 31 high-grade ovarian serous carcinomas, the most lethal gynecologic neoplastic disease in women. We identified an amplicon at 19p13.12 in 6 of 31 (19.5%) ovarian high-grade serous carcinomas. This amplification was validated by digital karyotyping, quantitative real-time PCR, and dual-color fluorescence *in situ* hybridization (FISH) analysis. Comprehensive mRNA expression analysis of all 34 genes within the minimal amplicon identified *Notch3* as the gene that showed most significant overexpression in amplified tumors compared with nonamplified tumors. Furthermore, *Notch3* DNA copy number is positively correlated with *Notch3* protein expression based on parallel immunohistochemistry and FISH studies in 111 high-grade tumors. Inactivation of *Notch3* by both γ -secretase inhibitor and *Notch3*-specific small interfering RNA suppressed cell proliferation and induced apoptosis in the cell lines that overexpressed *Notch3* but not in those with minimal amount of *Notch3* expression. These results indicate that *Notch3* is required for proliferation and survival of *Notch3*-amplified tumors and inactivation of *Notch3* can be a potential therapeutic approach for ovarian carcinomas. (Cancer Res 2006; 66(12): 6312-8)

Introduction

Gene amplification is one of the key mechanisms in activating oncogenes in human cancer (1). Ovarian cancer is the most malignant gynecologic neoplasm. Each year, ~16,000 women will succumb to this disease. In ovarian carcinomas, amplifications of *cyclin E1* (2), *Her2/neu* (3), *AKT2* (4), *L-Myc* (5), and *Rsf-1* (6) have been reported. Because these amplifications occur only in a subset of tumors, it is expected that additional oncogenic amplifications will be identified. Recent developments of molecular genetic techniques that allow a genome-wide exploration of DNA copy number in cancer have provided investigators unprecedented opportunities to analyze cancer genome in great details. For example, single nucleotide polymorphism (SNP) array has been recently shown as an effective tool in discovering amplified chromosomal regions (7, 8). In the current study, we did SNP array analysis on 31 high-grade ovarian serous carcinomas purified

from fresh clinical samples and identified a chromosomal region at 19p13.12 that is frequently amplified. One of the genes within this amplicon, *Notch3*, showed the most significant correlation between gene copy numbers and transcript expression in ovarian cancer, suggesting that *Notch3* is a candidate oncogene in the chr19p13.12 amplicon.

Materials and Methods

Tumor specimens. For SNP arrays, tissue samples, including 31 high-grade and 7 low-grade ovarian serous carcinomas, were obtained from the department of pathology at the Johns Hopkins Hospital and the Norwegian Radium Hospital in Norway. Tumor cells were affinity purified by anti-EPCAM-conjugated beads. In addition, genomic DNA from 13 normal ovarian tissues was prepared for controls. For quantitative reverse transcription-PCR (RT-PCR), 89 frozen tumor tissues were used to extract RNA, and nine normal ovarian tissues were also included as controls. Acquisition of tissue specimens and clinical information was approved by an institutional review board (Johns Hopkins University) or by the Regional Ethics Committee (Norway).

SNP array. SNPs were genotyped using 10K arrays (Affymetrix, Santa Clara, CA) in the Microarray Core Facility at the Dana-Farber Cancer Institute (Boston, MA). A detailed protocol is available at the Core center web page.⁴ Briefly, genomic DNA was cleaved with the restriction enzyme, *XbaI*, ligated with linkers, followed by PCR amplification. The PCR products were purified and then digested with DNaseI to a size ranging from 250 to 2,000 bp. Fragmented PCR products were then labeled with biotin and hybridized to the array. Arrays were then washed on the Affymetrix fluidics stations. The bound DNA was then fluorescently labeled using streptavidin-phycoerythrin conjugates and scanned using the Gene Chip Scanner 3000.

dChip software version 1.3 was used to analyze the SNP array data as described previously (7, 8). Data was normalized to a baseline array with median signal intensity at the probe intensity level using the invariant set normalization method. A model-based (PM/MM) method was used to obtain the signal values for each SNP in each array. Signal values for each SNP were compared with the average intensities from 13 normal samples. To infer the DNA copy number from the raw signal data, we used the Hidden-Markov model (7) based on the assumption of diploidy for normal samples. Mapping information of SNP locations and cytogenetic band were based on curation of Affymetrix and University of California Santa Cruz hg15. A cutoff of >2.8 copies in more than three consecutive SNPs was defined as amplification.

Digital karyotyping. Purified carcinoma cells as described in the SNP array method were used to generate digital karyotyping library as previously described (9). Approximate 120,000 genomic tags were obtained for each digital karyotyping library. After removing the nucleotide repeats in human genome, the average of filtered tags was 66,000 for each library. We set up a window size of 300 (300 virtual tags) for the analysis in this study. Based on Monte Carlo simulation, the variables used in this study can reliably detect >0.6 Mb amplicon with >5-fold amplification with >99% sensitivity and 100% positive predictive value.

Note: Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

Requests for reprints: Tian-Li Wang, Departments of Gynecology/Obstetrics and Oncology, Johns Hopkins University School of Medicine, CRBII, 1550 East Jefferson Street, Room 306, Baltimore, MD 21231. Phone: 410-502-7774; Fax: 410-502-7943; E-mail: tlw@jhmi.edu.

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⁴ <http://chip.dfci.harvard.edu/lab/services.php>.

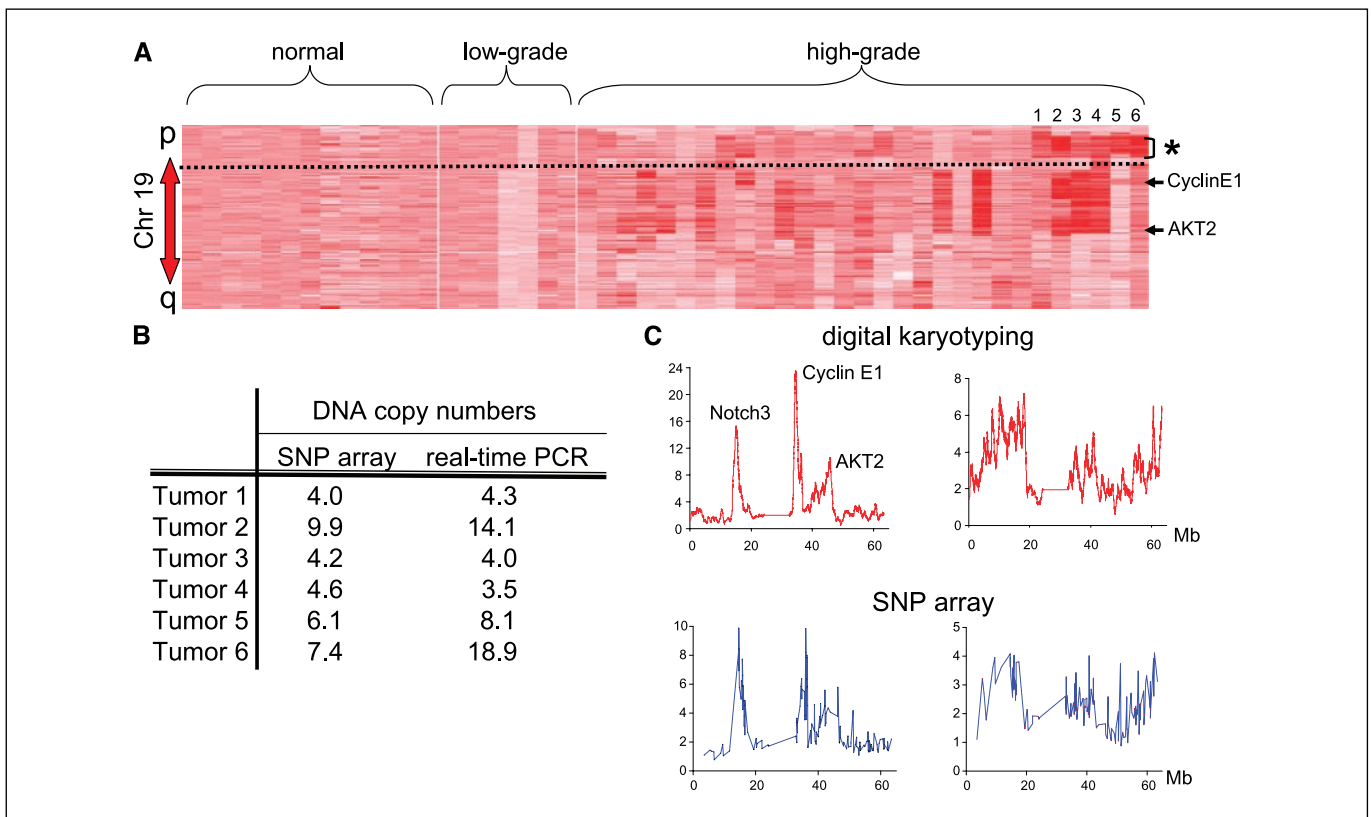


Figure 1. Identification of chr19p13.12 amplification in high-grade ovarian serous carcinomas. **A**, SNP array analysis shows several distinct amplicons on chromosome 19 (top to bottom, p to q arm) including the *cyclin E1* and *AKT2* loci and a novel amplicon at chr19p13.12 (bracket). An increase in DNA copy number is indicated by a gradient in color with 0 copy in white and five or more copies in red. Each column represents an individual tumor sample. **B**, quantitative real-time PCR on genomic DNA validates the amplification on the six tumors with chr19p13.12 amplification identified by the SNP array analysis. **C**, comparison of DNA copy number using SNP array and digital karyotyping on chromosome 19. The data of SNP array is compared with that of digital karyotyping analysis on two representative specimens. *Left*, tumor 2, which contains a high copy number amplification at chr19p13.12. *Right*, tumor 1, which contains a low copy number gain at chr19p13.12. Both SNP array and digital karyotyping analyses show a similar pattern of DNA copy number alterations.

Fluorescence *in situ* hybridization and quantitative real-time PCR.

BAC clones (RP11-937H1 and RP11-319O10) containing the genomic sequences of the 19p13.12 amplicon at 15.00 to 15.25 Mb were purchased from Bacpac Resources (Children's Hospital, Oakland, CA). BAC clone (RP11-752A21), located at 19q13.42 (59.26-59.46 Mb), was used to generate the reference probe. The method for fluorescence *in situ* hybridization (FISH) has been detailed in a previous report (6).

Relative gene expression and genomic amplification levels were measured by quantitative real-time PCR using methods previously described (9, 10). PCR primers were designed using the Primer 3 program and the nucleotide sequences of the primers for determining transcript expression were listed in Supplementary Table S1. The primer sequences to determine the 19p13.12 genomic amplification were 5'-GCCTGTGGCTGAAAATTAAGG-3' and 5'-TCAATGTCCACCTCGCAATAG-3'.

Immunohistochemistry. A rabbit polyclonal anti-Notch3 antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and was used in the immunohistochemistry. An EnVision+System peroxidase kit (DAKO, Carpinteria, CA) was used for staining following the protocol provided by the manufacturer. Tissue microarrays (triplicate 1.5 mm cores from each specimen) including 111 high-grade serous carcinomas and 10 normal ovaries were used to facilitate immunohistochemistry. Immunointensity was scored as negative (0), negligible (1+), moderate (2+), and intense (3+), and two investigators independently scored all the samples. For discordant cases, a third investigator scored and the final intensity score was determined by the majority scores.

Cell proliferation and apoptosis assays. Cells were grown in 96-well plates at a density of 4,000 per well. Type 1 γ -secretase inhibitor was purchased from Calbiochem (San Diego, CA) and was dissolved in DMSO

as 4 mmol/L stock solution. Cells were treated with γ -secretase inhibitor with DMSO final concentration <0.1%. Notch3-specific small interfering RNA (siRNA) (rGrUrCrArArUrGrUrUrCrArCrUrUrCrGrCrArGrUrU and rGrCrGrUrGrGrArUrUrCrGrGrArCrArGrUrCrUrGrArGrArGrGrG) and control siRNA that targets the *Luciferase* gene (rGrArUrUrArArUrCrUrUrCrUrArGrCrGrArCrUrGrCrUrUrCrGrC) were synthesized by Integrated DNA Technologies (Coralville, IA). Cells were treated with siRNA at a final concentration of 200 nmol/L.

Cell number was measured by the fluorescence intensity of SYBR green I nucleic acid gel stain (Molecular Probes, Eugene, OR) using a fluorescence microplate reader (Fluostar from BMG, Durham, NC). Data was determined from five replicates and was expressed as the percentage of the inhibitor or Notch3 siRNA-treated cells versus DMSO or control siRNA-treated cells. BrdUrd uptake and staining were done using a cell proliferation kit (Amersham, Buckinghamshire, England, United Kingdom) and apoptotic cells were detected using an Annexin V staining kit (BioVision, Mountain View, CA). The percentage of BrdUrd-positive and Annexin V-positive cells was determined by counting ~400 cells from each well in 96-well plates. The data was expressed as mean \pm 1 SD from triplicates.

Results and Discussion

Amplification of chromosome 19 in ovarian serous carcinomas. SNP arrays were used to search for genome-wide DNA copy number alterations in 31 high-grade and 7 low-grade ovarian serous carcinomas. In addition, 13 normal ovarian tissues were analyzed as controls. We found several distinct amplifications on

chromosome 19 specific to high-grade serous carcinomas. Among them, amplification of the *cyclin E1* locus was present in 10 of 31 (32.2%) samples and amplification of *AKT2* locus in 9 of 31 (29%) samples. Both *cyclin E1* and *AKT2* have been previously reported as potential oncogenes that are frequently amplified in ovarian cancer. More importantly, a novel amplification on chr19p13.12 was identified in 6 of 31 (19.5%) high-grade carcinomas. The peak copy number changes in these six amplified tumors ranged from four to nine copies based on SNP array analysis (Fig. 1A and B). In contrast to high-grade serous carcinomas, no evidence of amplification at 19p13.12, or *cyclin E1* or *AKT2* loci could be detected in low-grade ovarian tumors or normal ovarian tissues.

Three independent methods were used to validate the 19p13.12 amplification. First, digital karyotyping was done on a tumor with low level of gain (four copies) and a tumor with high level of amplification (nine copies) based on SNP array analysis. Digital karyotyping is a recently developed genome-wide technology that allows a precise measurement of DNA copy number at high resolution (9). The method has been used to identify new amplicons in human cancer (6, 11–13) and using digital karyotyping, we found similar location and amplitude of these amplicons detected by SNP arrays (Fig. 1C). Second, quantitative real-time PCR was used to measure the copy number of the predicted amplification in all of the six tumors (Fig. 1B). The amplification levels based on the SNP arrays were generally in agreement with quantitative PCR but with an underestimation of copy number in

those samples with high amplitude of amplifications. This could be due to the saturation of probe hybridization signal associated with array platform. Third, dual-color FISH analysis was done on all six amplified tumors using a BAC clone located at the 19p13.12 amplicon and a BAC clone located at the 19q as the reference. All of the six cases showed increased target signals compared with the reference signals. Among them, four cases showed a pattern of chromosomal gain and two cases showed a pattern of homogeneously staining region. Taken together, the above results confirmed amplification of 19p13.12 in high-grade ovarian serous carcinomas.

Notch3 as the candidate cancer-related gene in the 19p13.12 amplicon. To select the most promising tumor-associated gene within the minimal amplicon, we aligned the amplicons from these six tumors and delineated a common region of amplification (minimal amplicon), which spanned from 14.60 to 16.47 Mb on chromosome 19p and contained 34 genes (Fig. 2). To identify the candidate-amplified tumor-associated gene within the amplicon, we correlated the gene copy number and gene expression levels for all 34 genes based on the rationale that a tumor-driving gene, when amplified, should overexpress to activate its tumorigenic pathway, whereas coamplified “passenger” genes that are not related to tumor development may or may not do so (14). This approach can be useful to narrow down the candidate gene lists, although some amplification events may not necessarily have overexpression of the gene of interest. The mRNA levels were measured using quantitative RT-PCR on five high-grade carcinomas with 19p13.12

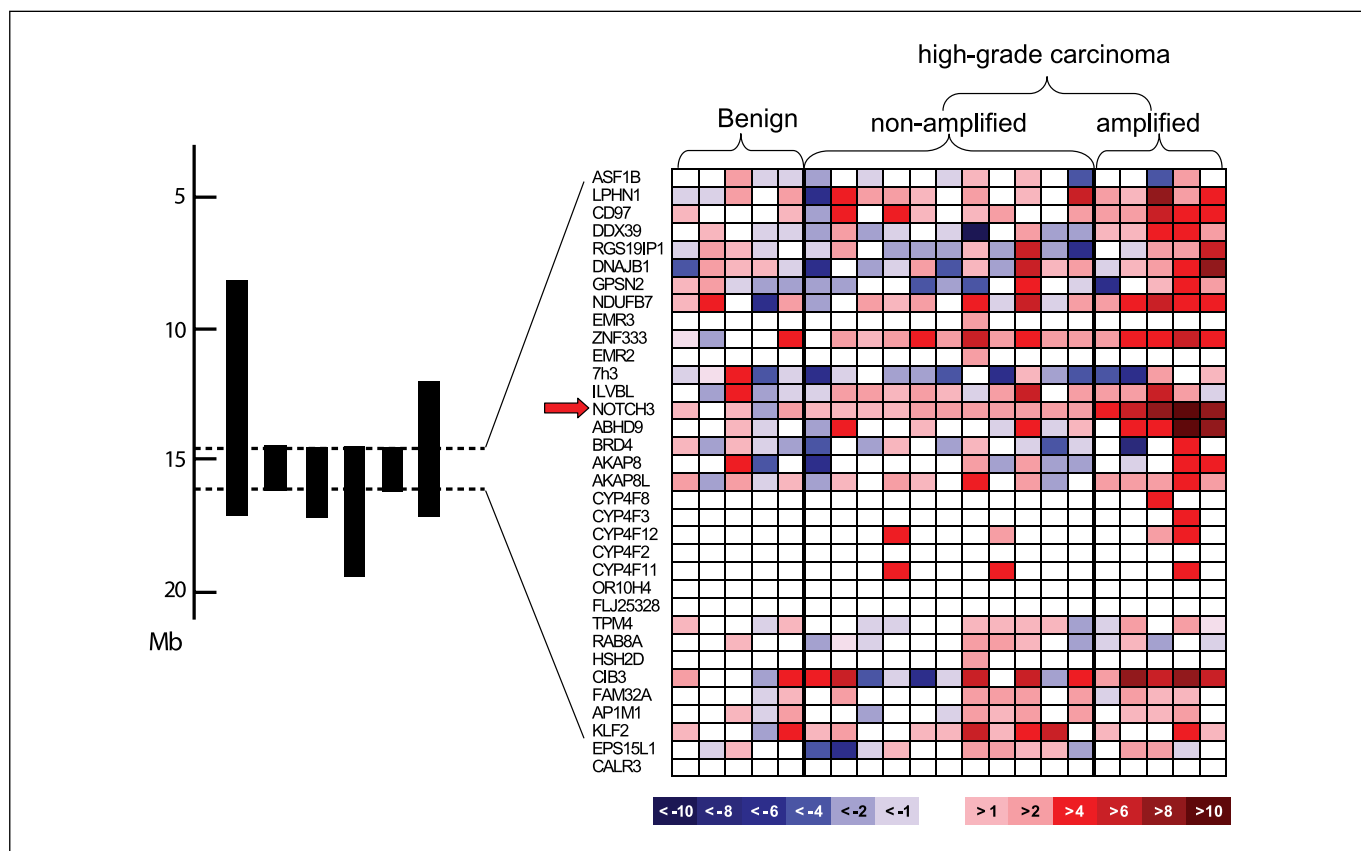


Figure 2. Gene expression analysis of the 19p13.12 amplicon in ovarian tumors. *Left*, alignment of the amplicons from the 19p13.12-amplified tumors reveals a common region of amplification spanning from 14.60 to 16.47 Mb at chromosome 19p. *Right*, quantitative real-time PCR was done for all 34 genes located within the minimal amplicon in high-grade serous carcinomas with or without 19p13.12 amplification and benign OSE cells were used as controls. The expression level of each gene (*top to bottom*, centromeric to telomeric) in individual specimen is shown as a pseudocolor gradient based on the relative expression level of a given specimen to the average value derived from five benign OSE samples.

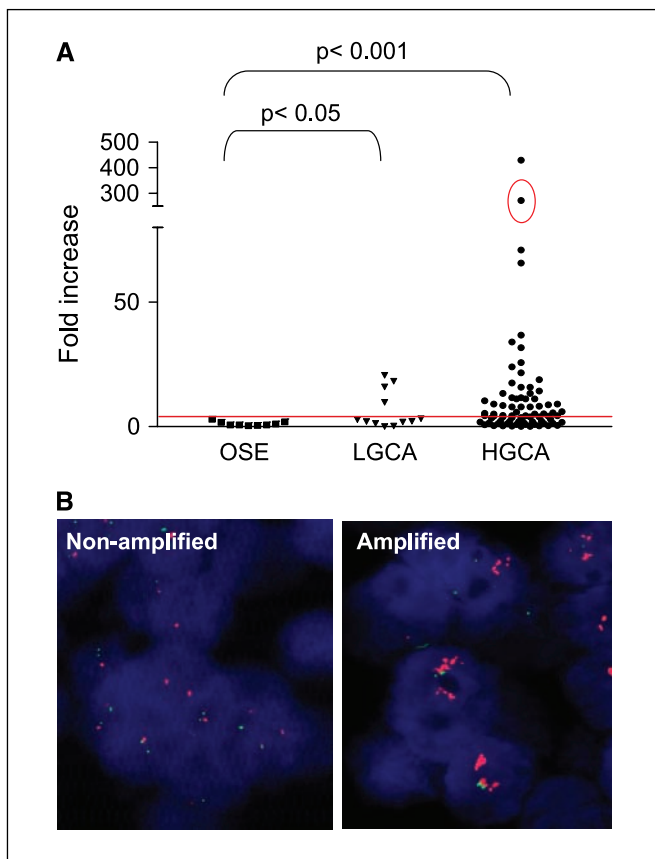


Figure 3. Overexpression and amplification of Notch3 in high-grade ovarian carcinomas. *A*, mRNA expression level of Notch3 for each specimen is measured by quantitative RT-PCR and is expressed as fold increase relative to the average value derived from nine OSE samples. Each symbol represents one specimen. The sample with circle harbored 19p13.12 amplification, which is shown in *(B)*. *B*, FISH analysis shows a homogeneously stained region in an amplified tumor (*right*) with a high level of mRNA expression. A nonamplified tumor (*left*) is also shown as a control.

amplification, 11 high-grade tumors without such amplification, and five benign ovarian tissues including four OSE samples and a benign ovarian cyst. The expression levels for each gene were normalized to the average value of benign tissues (Fig. 2). Mann-Whitney *U* test was used to compute and compare the difference in gene expression levels between the 19p13.12 amplified versus nonamplified high-grade carcinomas. Among the 34 genes within the minimal amplicon, *Notch3* showed the most consistent and significant up-regulation in 19p13.12-amplified tumors compared with nonamplified tumors ($P = 0.0018$). Other genes in the amplicon, such as *DDX39* and *ADHD9*, also showed a significant overexpression in the amplified versus nonamplified tumors ($P = 0.01$ and $P = 0.02$, respectively). It is plausible that these genes also contribute to the tumorigenesis in ovarian cancer. In this study, we selected *Notch3* for further characterization because it showed the most significant *P* value. We further correlated *Notch3* DNA and mRNA copy number in a set of 31 samples in which both tissues and RNA samples were available for FISH and quantitative RT-PCR analyses. Our data showed a moderate positive correlation of these two variables with a correlation coefficient of 0.481 (Spearman rank-order test, $P < 0.05$).

The above results, together with previous reports showing that *Notch3* participates in both development and oncogenesis, suggest

that *Notch3* is a candidate "driving" gene in the 19p13.12 amplicon. Therefore, *Notch3* was prioritized for further characterization in this study. We then did quantitative RT-PCR on a large panel of ovarian serous tumors to determine *Notch3* mRNA levels. As shown in Fig. 3*A*, *Notch3* was overexpressed in 66% (51 of 77) of high-grade tumors, but only in 33% (4 of 12) of low-grade tumor compared with normal ovarian surface epithelium (OSE). In addition, the top five tumors with the highest *Notch3* mRNA expression harbored 19p13.12 amplification (Fig. 3*B*), further supporting *Notch3* as a candidate amplified gene that is important in tumor development. Mann-Whitney *U* test showed that there was a statistically significant difference in the levels of *Notch3* expression between OSE and high-grade serous carcinomas ($P < 0.001$) and between OSE and low-grade serous carcinomas ($P < 0.05$). However, there was no significant difference between low-grade and high-grade carcinomas ($P = 0.3187$).

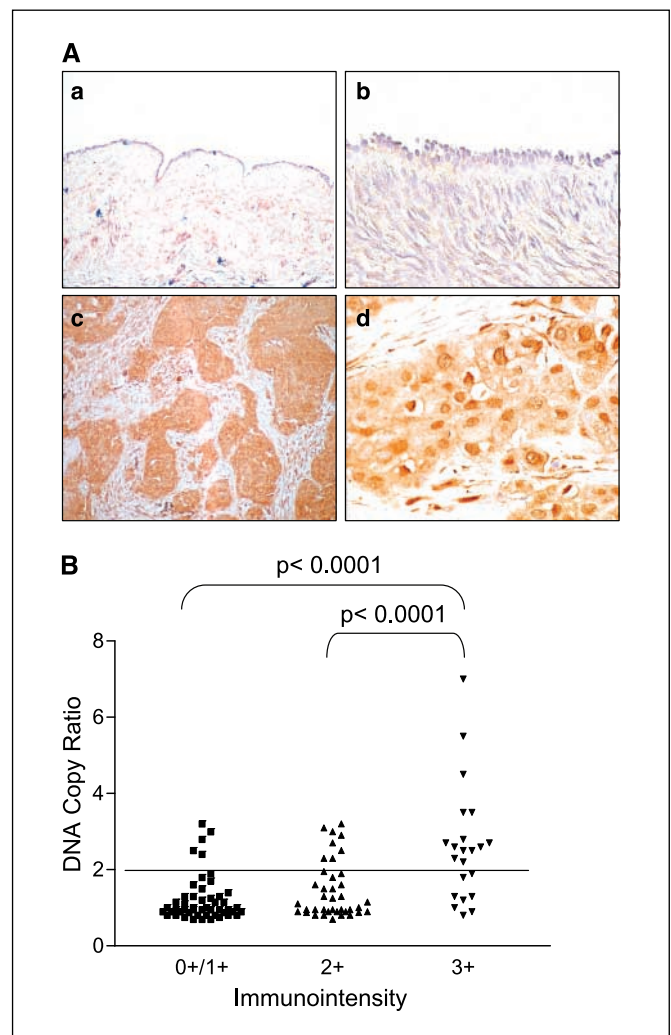


Figure 4. Immunoreactivity of Notch3 in ovarian tumors. *A*, immunoreactivity of Notch3 is not detectable in normal OSE (*a* and *b*) but is overexpressed in 55% of high-grade serous carcinoma. Immunolocalization of Notch3 is detected in both nucleus and cytoplasm in the tumor cells (*c* and *d*). *B*, correlation of Notch3 immunointensity and DNA copy number ratio in high-grade serous carcinomas. The intensity of Notch3 immunoreactivity positively correlates with the Notch3 DNA copy ratio based on FISH analysis. Furthermore, there is a statistically significant difference between tumors with intense staining intensity (3+) and those without (Mann-Whitney *U* test).

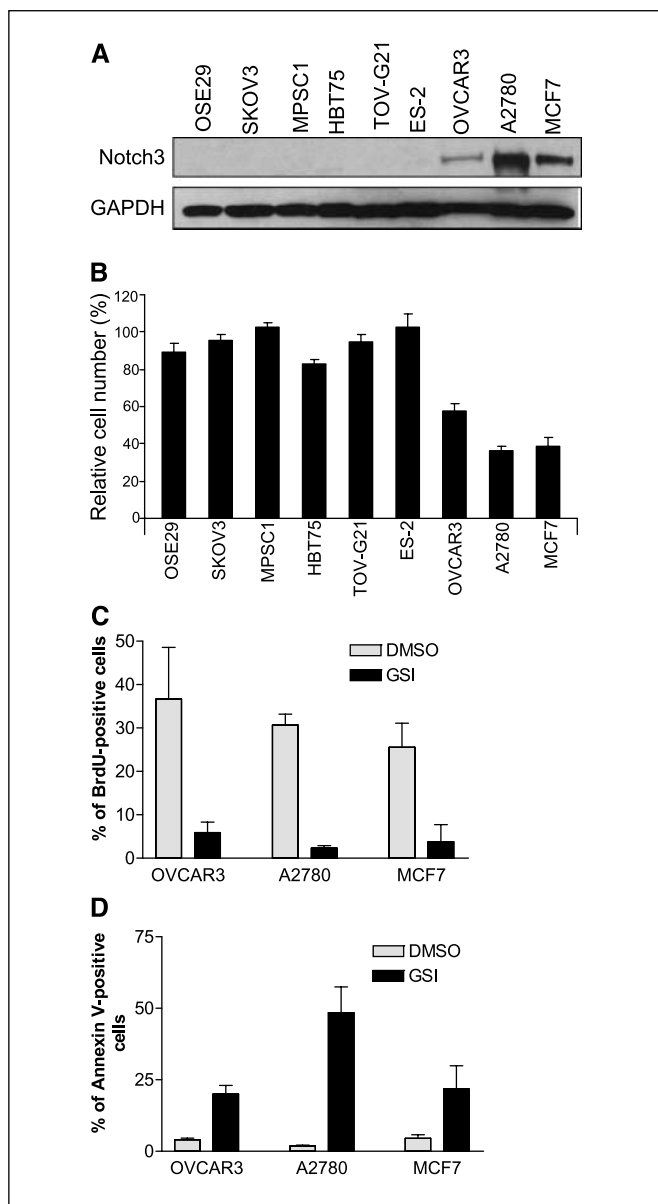


Figure 5. Effects of γ -secretase inhibitor on cell proliferation and apoptosis in cell lines. *A*, Western blot analysis shows a higher expression level of Notch3 protein in OVCAR3, A2780, and MCF-7 cells compared with the other cell lines in which the Notch3 protein is not detectable. *B*, γ -secretase inhibitor significantly reduces the cell number in cell lines with Notch3 overexpression, including OVCAR3, A2780, and MCF-7 compared with those without Notch3 overexpression. γ -Secretase inhibitor decreases DNA synthesis (*C*) and increases apoptosis (*D*) in OVCAR, A2780, and MCF-7 cells based on measurement of BrdUrd uptake and Annexin V labeling assays, respectively.

Immunohistochemistry and FISH were done in parallel on 111 tumor samples to correlate protein expression and *Notch3* DNA copy number. Notch3 immunoreactivity was detected in both nucleus and cytoplasm of tumor cells in 61 of 111 (55%) carcinomas but not in normal ovarian epithelial cells (Fig. 4A). Notch3 immunointensity was scored as negative (0), negligible (1+), moderate (2+), and intense (3+) and was correlated with *Notch3* DNA copy number ratio based on FISH analysis (Fig. 4B). Specifically, the intensity of Notch3 staining positively correlated with the *Notch3* DNA copy ratio (Spearman rank-order correlation coefficient = 0.4). Furthermore, there is statistically significant

difference between tumors with intense staining intensity (3+) and those without (intensity score = 0, 1+, and 2+; Mann-Whitney *U* test, $P < 0.0001$). We observed that 8 of 22 carcinomas are with Notch3 overexpression (3+) but without *Notch3* gene amplification (gene copy ratio ≤ 2). This finding implies that in addition to gene amplification, mechanisms such as epigenetic activation of the *Notch3* promoter in response to environmental cues may contribute to Notch overexpressing in those tumors. Although a positive correlation of *Notch3* gene copy ratio and protein expression was observed, there are some tumors (5 of 50 tumors)

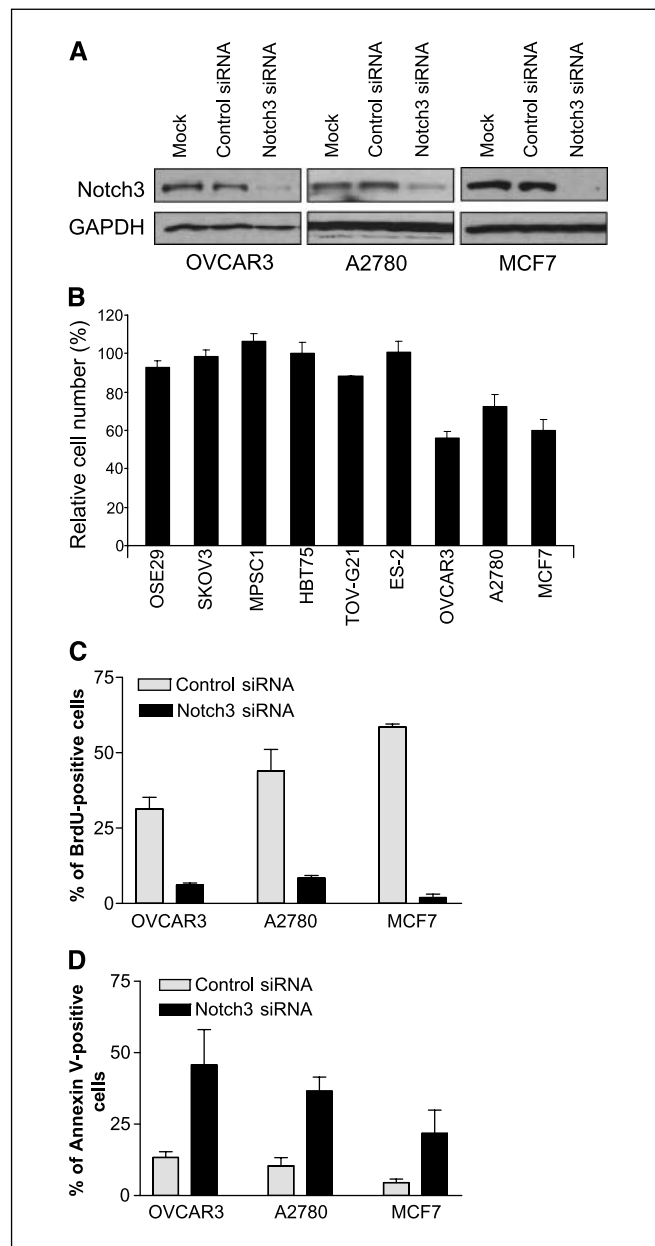


Figure 6. Effects of Notch3 knockdown on cell proliferation and apoptosis in cell lines. *A*, Western blot analysis shows a significant reduction of Notch3 protein in Notch3 siRNA-treated cells compared with the mock or control siRNA-treated cells. *B*, Notch3 siRNA significantly reduces the cell number in cell lines with Notch3 overexpression, including OVCAR3, A2780, and MCF-7 compared with those without. Treatment with Notch3 siRNA decreases DNA synthesis as measured by BrdUrd uptake (*C*) and increases apoptosis as measured by Annexin V labeling (*D*) in OVCAR3, A2780, and MCF-7 cells.

with *Notch3* DNA copy ratio >2 but with only weak Notch3 protein expression (0/1+). This result suggests that these carcinomas may use other oncogene(s) that resides on the same chromosomal arm as the *Notch3* locus to promote tumor progression in ovarian serous carcinomas.

Notch receptors participate in signal transduction by translocating their cytoplasmic domain to the nucleus where it activates an array of downstream effectors that can play important roles in cell proliferation and survival (15). The association of genetic changes in *Notch3* and human cancer has been recently established in lung carcinoma. Translocation of t(15;19) was identified in non-small-cell lung cancer cell lines and the breakpoint has been mapped to 50 bp upstream of the *Notch3* locus. The translocation of chromosome 19p was found to correlate with overexpression of Notch3 full-length mRNA (16). Our data here provide new evidence that besides translocation, gene amplification is another mechanism to activate Notch3 in human cancer.

The genetic findings are supported by mouse models. For example, expression of the intracellular domain (a constitutively active form) of Notch3 in mouse thymus induced T-cell leukemia/lymphoma (17). Furthermore, constitutive expression of activated Notch3 in the central nervous system initiates the formation of brain tumors in the choroid plexus in mice (18), suggesting that deregulation of Notch3 plays a role in neoplastic transformation.

Functional analysis of Notch3 expression. To determine if Notch3 is essential for cell growth and survival in cell lines that overexpress Notch3, we used γ -secretase inhibitor 1, which prevents the activation of Notch3 by inhibiting the proteolysis and translocation of Notch3 cytoplasmic domain to the nucleus. This compound has been shown to be a potent and specific inhibitor of Notch pathway (19, 20). γ -Secretase inhibitor was applied to the culture medium in nine cell lines, including three cancer cell lines with Notch3 overexpression (OVCAR3, A2780, and MCF7), an immortalized OSE cell line (OSE 29), and five cancer cell lines (SKOV3, MPSC1, HTB75, TOV-G21, and ES-2) without Notch3 overexpression (Fig. 5A). We first determined the concentration of γ -secretase inhibitor to be used and found that the IC₅₀ was lowest in OVCAR3 (1 μ mol/L). Therefore, we treated different cell lines with inhibitor at 1 μ mol/L in culture and found that there was a substantial reduction in cell number of OVCAR3, A2780, and MCF7 cells that overexpressed Notch3 compared with other cell lines that did not have Notch3 overexpression (Fig. 5B, $P < 0.001$, Student's t test). To assess the mechanisms underlying the growth inhibition by the γ -secretase inhibitor in OVCAR3, A2780, and MCF-7 cells, we measured the percentage of BrdUrd-labeled cells for cellular proliferation and Annexin V-labeled cells for apoptosis (Fig. 5C and D). We found that γ -secretase inhibitor significantly reduced cellular proliferation and induced apoptosis in all three cell lines with Notch3 overexpression compared with the DMSO controls ($P < 0.001$, Student's t test). siRNA was used to knock down the expression of Notch3 in the same nine cell lines used for

the γ -secretase inhibitor assay. The knockdown effect of siRNA was shown by Western blot (Fig. 6A). siRNA treatment significantly reduced the Notch3 protein expression compared with the mock or control siRNA-treated groups. Similar to the effects of γ -secretase inhibitor, Notch3 siRNA reduced cell number most significantly in OVCAR3, A2780, and MCF7 cells, which overexpressed Notch3 compared with the other cell lines (Fig. 6B, $P < 0.001$, Student's t test). The BrdUrd-positive cells decreased and the Annexin V-labeled cells increased in Notch3 siRNA-treated cells compared with control siRNA-treated cells (Fig. 6C and D, $P < 0.001$, Student's t test).

Our *in vitro* data to inactivate Notch3 by γ -secretase inhibitor and siRNA may have clinical implications for ovarian cancer patients and suggest that Notch3 can be a candidate therapeutic target. γ -Secretase inhibitors have been studied in the past several years as a potential therapeutic intervention in Alzheimer's disease. Very recently, γ -secretase inhibitors have been shown to inhibit the epithelial cell proliferation and induce goblet cell differentiation in intestinal adenomas *Apc*^{-/-} (min) mice (20). Furthermore, γ -secretase was shown to be able to inhibit the growth of Kaposi's sarcoma cells in mouse tumor model (19). Therefore, with the promising effects at both *in vitro* and *in vivo* systems, γ -secretase inhibitors can be used as new target-based therapy for those tumors with Notch3 activation.

The current study suggests that *Notch3* is a strong candidate oncogene among the genes within the ch19p13.12 amplicon in ovarian carcinomas. This is because *Notch3* gene shows a high correlation of gene amplification and overexpression and is functionally essential for tumor growth and survival. Although the above represents our preferred interpretation, other alternative interpretations should be pointed out. For example, *Notch3* may not be the only gene with high correlation of DNA copy number and gene expression level after analyzing a large series of amplified and nonamplified tumors. It is possible that other coamplified gene(s) within the *Notch3* amplicon also plays a role in tumorigenesis and they may cooperate with Notch3 in propelling tumor progression.

In conclusion, we have identified *Notch3* as a candidate amplified oncogene that overexpressed in 66% of ovarian serous carcinomas. Our findings suggest that *Notch3* amplification may play an important role in the development of ovarian carcinomas; moreover, these findings provide a rationale for future development of Notch3-based therapy for ovarian cancer.

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