学 位 論 文 の 要 旨

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学　位　論　文　名　Gene Amplification *CCNE1* is Related to Poor Survival and Potential Therapeutic Target in Ovarian Cancer

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論 文 内 容 の 要 旨

INTRODUCTION

Ovarian carcinoma is the most lethal gynecological malignancy in American women and is the most lethal gynecological cancer in Japan. Incidence rate has increased dramatically in the last decade. In greater than 70% of patients, there is evidence of tumor dissemination beyond the ovaries at diagnosis. In these cases, combined treatment with surgery and chemotherapy is necessary. First-line chemotherapy with platinum drugs and taxanes yields a response rate of over 80%, but almost all patients relapse. Thus, there is an initial, preclinical need to improve understanding of the molecular pathways underlying ovarian carcinogenesis.

Our previous genome-wide analysis, single nucleotide polymorphism array identified *CCNE1* (*Cyclin E1*) as the most frequent amplified gene in ovarian serous carcinomas. High levels of CCNE1 protein, an activating subunit of cyclin dependent kinase 2 (CDK2), are often observed in patients with ovarian cancer. Deregulation of cell cycle control is thought to be a prerequisite in tumor development, and several studies have shown an accelerated entry into S phase because of constitutive expression of CCNE1. Furthermore, CCNE1 is able to induce chromosome instability by inappropriate initiation of DNA replication and centrosome duplication. Several studies consistently have demonstrated that CCNE1 is associated with disease progression in various malignancies and is associated clinically with poor prognosis in patients with breast, bladder, and colorectal carcinoma. Most such studies analyzed ovarian carcinoma only through protein expression using immunohistochemistry. Gene amplification is an important mechanism that allows cancer cells to increase expression of driver genes, such as oncogenes involved in growth regulation and genes responsible for drug resistance. Therefore, detection of gene amplification in tumors may be of diagnostic, prognostic, and/or therapeutic relevance for patient management.

This study examined the clinical significance of *CCNE1* (*Cyclin E1*) amplification and assessed whether CCNE1 is a potential target in ovarian cancer.

MATERIALS AND METHODS

Formalin-fixed, paraffin-embedded tissue samples of 88 ovarian cancers, including 45 serous carcinomas, 10 mucinous carcinomas, 10 clear cell carcinomas, and 23 endometrioid carcinomas, were used in this study. Samples were obtained from the Department of Obstetrics and Gynecology at the Shimane University Hospital. Diagnosis was based on conventional morphological examination of sections stained with hematoxylin and eosin, and tumors were classified according to the World Health Organization classification. Tumor staging was performed according to the International Federation of Gynecology and Obstetrics (FIGO) classification. All patients were primarily treated with cytoreductive surgery and adjuvant platinum and taxane chemotherapy. The Shimane University Institutional Review Board approved the acquisition of tumor tissues and written informed consent was obtained from all subjects.

Bacterial artificial chromosome (BAC) clones (RP11-345J21 and CTD-3005A16) containing the genomic sequences of the 19q12 amplicon at 15.00 to 15.25 Mb were purchased from Bacpac Resources and Invitrogen. BAC clones located at Chr2q11.2 (eg, RP11-127K18 and RP11-629A22) or at Ch19P12 (CTD-2518O18) were used to generate reference probes. RP11-127K18, RP11-629A22, and CTD-2518O18 were labeled by nick translation with biotin-dUTP; RP11-345J21, and CTD-3005A16 were labeled similarly with digoxigenin-dUTP. To detect biotin-labeled and digoxigenin-labeled signals, slides were first incubated with FITC-avidin and an anti-digoxigenin mouse antibody. Slides were subsequently incubated with a biotinylated anti-avidin antibody and tetramethylrhodamine B isothiocyanate (TRITC)-conjugated rabbit anti-mouse antibody. The final incubation was with FITC-avidin and TRITC-conjugated goat anti-rabbit antibody. Approximately 100 tumor cells were examined for each specimen and the numbers of fluorescent signals within tumor cells from the *CCNE1* gene BAC probe and chromosome 2q11.2 or 19p12 reference BAC probe were recorded. Amplification of *CCNE1* was defined as a ratio of *CCNE1* BAC probe signals to chromosome 2 or chromosome 19 centromeric reference BAC probe signals of 2:1 or more.

Expression of CCNE1 and CDK2 was assessed by immunohistochemistry and/or Western blot analysis. The antibodies used in this study were a mouse monoclonal antibody that reacted with CCNE1 (Zymed) and a mouse monoclonal antibody that reacted with CDK2 (Abcam). Immunohistochemistry studies for CCNE1 and CDK2 were performed on tissue microarrays at a dilution of 1:250 or 1:50 followed by detection with the En Vision+ System using the peroxidase method. Slides for all samples were evaluated with a light microscope by 2 researchers; the

researchers were blind to clinicopathologic factors. The antibody staining intensity was then analyzed in glands and stroma using the HSCORE.

Overall survival was calculated from date of diagnosis to date of death or last follow-up. Survival data were plotted as Kaplan-Meier curves, and statistical significance was determined by the log-rank test. Multivariate prognostic analysis was performed using a Cox proportional hazards model. Data were censored when patients were lost to follow-up. The Pearson correlation coefficient test was used to examine statistical significance in differences between DNA copy number and immunohistochemical analysis values.

CCNE1 gene knockdown using silencing RNA and a *CCNE1* gene transfection system were used to asses CCNE1 function in tissue samples of ovarian cancer. Two silencing RNAs (siRNAs) that targeted CCNE1 were designed with the following sense sequences: UCAGUUGACAGUGUACAAUGCCUTT and UGACUUACAUGAAGUGCUACUGCCG. Control siRNA (luciferase siRNA) was purchased from IDT. Cells were transfected with siRNAs using oligofectamine. Two ovarian cancer cell lines ES2 and TOV-21G were transfected with plasmid vector pFLAG-N3 containing cDNA for *CCNE1*, using the Nucleofector II electroporator for generation of stable clones.

RESULTS AND DISCUSSION

CCNE1 gene amplification was identified in 18 (20.4%) of 88 ovarian carcinomas. *CCNE1* copy number significantly correlated with CCNE1 protein expression ($r = 0.522$, $p < 0.0001$). *CCNE1* amplification significantly correlated with shorter disease-free and overall survival ($p < 0.001$). There were nonsignificant trends between high protein expression and poor disease-free ($p = 0.2865$) and overall survival ($p = 0.1248$). Multivariate analysis showed gene amplification was an independent prognostic factor for disease-free and overall survival after standard platinum–taxane chemotherapy ($p = 0.0274$, $p = 0.0023$).

Profound growth inhibition and apoptosis were observed in silencing RNA–treated cancer cells with gene amplification compared with results in cancer cells with CCNE1 moderate-expression without gene amplification or with low CCNE1 expression. *CCNE1* overexpression stimulated proliferation in ovarian cancer cell lines ES2 and TOV-21G, which have lower endogenous CCNE1 expression.

CONCLUSION

These findings indicate that *CCNE1* overexpression is critical to growth and survival of ovarian cancer tumors with *CCNE1* gene amplification. Furthermore, they suggest that *CCNE1* silencing RNA-induced phenotypes depend on amplification status of ovarian cancers. Therefore, CCNE1-targeted therapy may benefit ovarian cancer patients with *CCNE1* amplification.

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論文審査の結果の要旨

卵巣がんは婦人科系がんの中で、乳がんに次ぎ２番目に罹患率が高く、増加傾向にある。卵巣がんの初期には自覚症状に乏しく、約半数の症例はⅢ・Ⅳ期の進行がんとして発見される。乳がんの５年相対生存率が９３％であるのに対し、卵巣がんは６１％と明らかに予後不良である。進行期卵巣がんに対する治療は、術後の残存腫瘍の有無が予後と相関することから腫瘍減量術を行い、シスプラチンとパクリタキセルを主体とした化学療法を行うのが標準である。化学療法の奏功率は８０％を超えるが、ほとんどすべての症例で再発する。そのため、新しい治療戦略の構築が喫緊の課題である。そこで申請者は卵巣がんのなかで一番頻度が高い漿液性腺がんの４０％以上で遺伝子増幅が観られる *CCNE1*（cyclin E1 をコードする遺伝子）に注目し基礎的検討を行った。卵巣がん患者組織ならびに卵巣がん細胞株を使用して、cyclin E1 の発現および *CCNE1* の遺伝子増幅をそれぞれ免疫染色および *in situ* ハイブリダイゼーション法で評価した。*CCNE1* の遺伝子増幅のある細胞株とない細胞株に対する *CCNE1* siRNA の効果を細胞増殖能、細胞周期への影響およびアポトーシスの観点から評価した。さらに、cyclin E1 を発現していない卵巣がん細胞株に *CCNE1* を安定導入させ、細胞増殖能を評価した。

CCNE1 の遺伝子増幅を認める場合は cyclin E1 の高発現を認めた。一方、*CCNE1* の遺伝子増幅がないにも関わらず cyclin E1 の高発現を認めた症例が存在しており、cyclin E1 の高発現には遺伝子増幅ばかりではなく転写レベルでの制御もあることを明らかにした。*CCNE1* の遺伝子増幅を認めた患者は明らかに予後不良であるが、cyclin E1 の高発現を認めただけでは予後不良とはならなかった。細胞株を用いた *CCNE1* のノックダウン実験から、*CCNE1* の遺伝子増幅に依存して、細胞周期の停止とアポトーシスの誘導により細胞増殖抑制を認めた。さらに、cyclin E1 を発現していない卵巣がん細胞株に *CCNE1* を安定導入した結果、細胞増殖能は増強した。これらの結果から、*CCNE1* は卵巣がんのドライバー遺伝子の一つであり、cyclin E1 と複合体を作り活性化するサイクリン依存性キナーゼ（cyclin-dependent kinase、以下 CDK）に対する阻害剤の臨床試験では *CCNE1* の遺伝子増幅が有益なバイオマーカーとなることが示唆された。卵巣がんの臨床において極めて重要な知見を示唆しており、重要な研究である。