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Original Paper

Messenger Ribonucleic Acid Expression of LH/hCG Receptor Gene in Human Ovarian Carcinomas

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The mRNA expression of luteinizing hormone (LH)/human chorionic gonadotropin (hCG) receptors was analysed by the RT-nested PCR method in five normal ovarian tissues, 62 ovarian tumours (5 benign, 7 borderline and 43 malignant epithelial tumours, 3 sex cord–stromal tumours and 4 germ cell tumours) and in 2 ovarian cancer cell lines. In normal ovaries, two cDNA fragments of different sizes were detected using primers designed to amplify a region including exon 9. Sequencing revealed that the larger fragment was derived from a full-length receptor, while the smaller fragment was a splice variant lacking exon 9. In ovarian tumours, the larger fragment of LH/hCG receptors was detected in 40% of the epithelial ovarian carcinomas, none of the germ cell tumours, all of the sex cord–stromal tumours and one of the 2 ovarian cancer cell lines. Immunohistochemistry confirmed the localisation of LH/hCG receptor protein in the tumour cells which correlated with mRNA expression. Patients with full-length LH/hCG receptors in carcinomas showed a better prognosis compared with those without the receptors. © **1997 Elsevier Science Ltd.**

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INTRODUCTION

LUTEINIZING HORMONE (LH) and human chorionic gonadotropin (hCG) bind to a common transmembrane glycoprotein receptor, which is a member of the G protein-coupled receptor family. The binding results in stimulation of adenyl cyclase and cAMP production [1]. The LH/CG receptor gene in pig [2], mouse [3], rat [4] and in human [5] has been cloned and in humans composed of 11 exons and 10 introns and its coding region is over 60 kilobases long [1]. Interestingly, aside from the full-length mRNA, various truncated forms have also been reported in pigs [2], rats [6] and humans [5]. These multiple species of LH/CG receptor mRNA are presumably derived from alternative splicing and/or differences in the degree of polyadenylation of the primary transcript [1]. Recently, it has been postulated that the splicing process is regulated in a tissue-specific manner [7, 8].

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The expression of LH/hCG receptor mRNA has been observed not only in the human ovary [5, 9], but also in non-gonadal tissues such as the fallopian tube [10], myometrial smooth muscle [11], thyroid [7], brain [12] and endometrium [13]. With regard to malignant neoplasms, endometrial carcinomas [13] and choriocarcinoma [14] have been reported to contain LH/hCG receptors. Pituitary gonadotropins have been suggested to be involved in ovarian carcinogenesis. The incidence of ovarian carcinoma increases around the perimenopausal period, when the serum levels of pituitary gonadotropins are elevated [15]. There have been reports of ovarian carcinoma arising in infertile women treated with gonadotropins [16]. However, whether ovarian cancer cells are gonadotropin-sensitive or insensitive, is still unknown because there have been conflicting reports regarding LH/hCG receptor status in ovarian tumour tissues as detected by ligand-binding assays [17–20]. The mRNA expression of LH/hCG receptor in ovarian carcinomas has not previously been reported.

In the present study, we analysed the expression pattern of mRNA of LH/hCG receptors in normal ovarian tissues

using RT-nested PCR. These structures of alternatively spliced mRNAs were determined by sequencing of subcloned PCR products. We screened 62 cases of epithelial and non-epithelial ovarian tumours and 2 ovarian cancer cell lines by the RT-nested PCR method. Localisation of LH/hCG receptor protein in ovarian carcinoma tissues was also analysed by immunohistochemistry. The correlation of LH/hCG receptor positivity with clinicopathology, expression of epidermal growth factor receptors (EGFR) and c-*erb* B-2 genes and patient survival was examined.

MATERIALS AND METHODS

Tissue samples and cell lines

Fresh surgical specimens of ovarian tumours were obtained from patients undergoing salpingo-oophorectomy. They consisted of 55 epithelial tumours (5 benign cystadenomas, 7 borderline tumours and 43 ovarian carcinomas) and 7 non-epithelial tumours (3 sex cord-stromal tumours [2 granulosa cell tumours, 1 thecoma], 4 germ cell tumours [3 dysgerminomas, 1 yolk sac tumour]). Clinical staging was performed according to the classification of the International Federation of Obstetrics and Gynecology [21]. The tissues for investigation were prepared carefully under a dissecting microscope to eliminate non-tumour components and stored at −80°C for subsequent analysis. If necessary, tumour tissues were obtained from collected 10 μm thick frozen sections, one of which was used to determine that the tumour component exceeded 80%. Patients with ovarian carcinoma were given postoperative cisplatin-based chemotherapy and followed for 1–59 months. Normal ovaries were obtained from 5 patients who underwent surgery for benign gynaecological diseases. Written informed consent was obtained from all patients according to the Guidelines (No. 90) of The Ethical Committee of Kyoto University Faculty of Medicine. Two ovarian cancer cell lines (SKOV-3, NIH:OVCAR-3) were purchased from the American Type Culture Collection.

RNA preparation and RT-nested PCR

Total RNA was isolated by the method of Chomczynski and Sacchi [22]. RT-nested PCR was performed according to the method of Lin and associates [13] with some modifications. Briefly, cDNA was prepared from 1 μg of total RNA by the random priming method using a First-Strand cDNA Synthesis Kit (Pharmacia-LKB, Uppsala, Sweden). The nucleotide sequences of primers for PCR were as follows: sense and antisense primers for first-round PCR were 5′-GCATCTGTAACACAGGCATC-3′ and 5′-CATCTGGTTCAGGAGCACAT-3′, respectively; sense and antisense primers for second-round PCR were 5′-GCAGAAGATGCACAATGGAG-3′ and 5′-CTCTCAGCAAGCATGGAAGA-3′, respectively. First-round PCR was carried out in a Thermal Cycler (Perkin-Elmer Cetus, Northwalk, Connecticut, U.S.A.) with a mixture consisting of cDNA derived from 50 ng of RNA, 8 pmol each of upstream and downstream primer, 200 μmol of dNTP, 0.1 unit of Taq DNA polymerase with reaction buffer (Takara Shuzo, Shiga, Japan) in a final volume of 10 μl. The PCR conditions consisted of denaturation for 5 min at 94°C, then 30 cycles of denaturation for 1 min at 94°C, annealing for 2 min at 55°C and extension for 1 min at 72°C, followed by a final extension reaction for 5 min at 72°C. One-tenth of the product of first-round PCR was used for second-round PCR, in which the PCR conditions were the same except nested primers were used [13]. Messenger RNA expressions of EGFR and c-*erb*B-2 were determined by RT–PCR as described previously [23].

Subcloning and sequencing of PCR products

The PCR products were subcloned into pUC18 using a TA Cloning Kit (Invitrogen, San Diego, California, U.S.A). At least 10 clones from each sample were screened by PCR and at least 3 clones containing inserts of the same size were sequenced. Sequencing reactions were performed using a Takara Taq Cycle Sequencing Kit (Takara Shuzo, Shiga, Japan) with FITC-labelled primers, and the products were analysed with an automatic DNA sequencer, DSQ-1000 System (Shimadzu, Kyoto, Japan). Sequencing analysis was performed in duplicate in upstream and downstream directions using common primers for pUC18.

Immunohistochemistry

The expression of LH/hCG receptors protein was analysed by immunohistochemistry in 7 cases of ovarian carcinomas. Rabbit polyclonal antibody raised against a synthetic peptide (15–38) of the rat luteal LH/CG receptor was kindly provided by Dr Patrick Roche from the Mayo Clinic (Rochester, Minnesota, U.S.A.). This antibody has been shown to cross-react with human LC/hCG receptor [13]. Formalin-fixed paraffin-embedded sections were deparaffinised, heated in a microwave oven for 10 min and treated with 0.3% hydrogen peroxide. Then they were incubated for 60 min at 37°C with a 1:250 dilution of receptor antibody. For a negative control, the first antibody was substituted by phosphate-buffered (PBS) solution. Immunostaining was performed by the avidin–biotin–peroxidase method using the Universal Rabbit System (Biomeda, Foster, California, U.S.A.).

Statistical analysis

The differences in the incidence of LH/hCG expression between groups were analysed using the chi-squared test. Differences in levels of EGFR and c-*erb*B-2 mRNAs according to the LH/hCG positivity were evaluated by the Mann–Whitney U test. The outcome of patients with or without LH/hCG receptors in ovarian carcinomas were compared by generalized Wilcoxon analysis, and the survival curves were generated using the Kaplan–Meier method.

RESULTS

RT-nested PCR analysis of LH/hCG receptor mRNA expression in normal and neoplastic ovarian tissues

In all normal ovarian tissues examined, RT-nested PCR amplified two different size fragments (Figure 1; lane 3), both of which corresponded to the splice variants reported previously [13]. In epithelial ovarian tumours, the sizes of amplified fragments were the same as those amplified from normal ovaries. However, the splicing pattern of mRNA in tumour tissues varied; the two variants were detected in 17 of the 43 (40%) ovarian carcinomas (Figure 1; lanes 6 and 7), 5 of the 7 (71%) borderline tumours and in 4 of the 5 (80%) cystadenomas; only the smaller fragment was detected in 4 (9%) carcinomas (Figure 1; lane 9), 2 (29%) borderline tumours and in one cystadenoma. Neither were detected in 22 (51%) carcinomas (Figure 1; lanes 4, 5, 8, and 11), including one case of stage III serous carcinoma

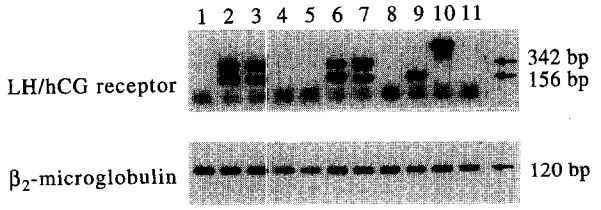

Figure 1. RT-nested PCR amplification of LH/hCG receptor and β2-microglobulin sequences. Lanes 1 and 2, ovarian cancer cell lines, SK-OV-3 and NIH:OVCAR-3, respectively. Lane 3, normal ovary. Lanes 4–11, ovarian carcinoma. An abnormally large fragment was amplified from an ovarian carcinoma (lane 10). The expression of $\beta_2$microglobulin (bottom) was used as a control for RT-PCR.

which exhibited an unusually large fragment (Figure 1; lane 10). This variation of splicing pattern of LH/hCG receptor mRNA was not significantly related to the clinical stage or histological type of ovarian carcinomas. Of the non-epithelial ovarian tumours, all the sex cord–stromal tumours (2 granulosa cell tumours and 1 thecoma) expressed both the fragments. In contrast, none of the germ cell tumours (3 dysgerminomas and 1 yolk sac tumour) expressed either the larger or the smaller size fragment. One ovarian cancer cell line (NIH:OVCAR-3) expressed both fragments (Figure 1; lane 2), whereas another (SKOV-3) (Figure 1; lane 1) expressed neither.

Structure of alternatively spliced LH/hCG receptor mRNA fragments

The PCR products from three normal ovaries expressing both fragments and from 3 representative cases (one expressed both fragments one only the smaller and one the unusual size fragments) of ovarian carcinomas were subcloned and sequenced. The structure of the larger fragment from the three normal ovaries and from one carcinoma corresponded to the full-length LH/hCG cDNA according to the published sequence [5]. The structure of the smaller fragment from three normal ovaries and one carcinoma was almost the same as the truncated variant reported previously [5], which lacked the region corresponding to exon 9. However, in all cases examined, one codon (TTA) was present at the exon 8–10 boundary, resulting in insertion of a leucine residue (Figure 2(A)). The abnormally large fragment in one case of stage III carcinoma lacked exon 9 and had a 536 nucleotide insertion at the putative exon 10–11 boundary (Figure 2(B)). Since the first codon of the inserted sequence was a stop codon (TAA), this unusual variant possibly produced a truncated protein lacking part of the extracellular domain, and the entire transmembrane

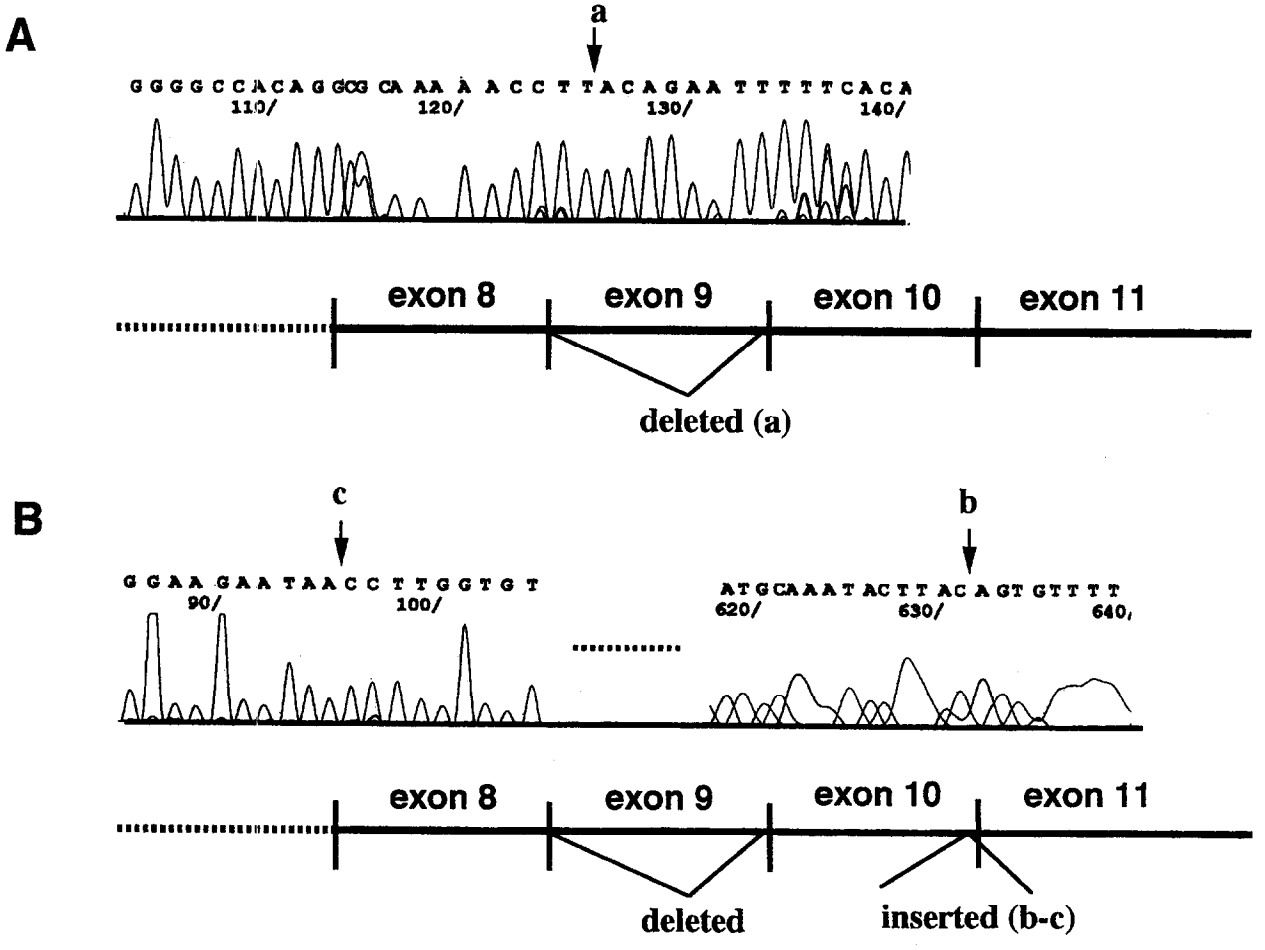

Figure 2. Structural analysis of LH/hCG receptor sequence variants. (A) Part of the sequence at the exon 8–10 boundary of the variant lacking exon 9. At the exon 8–10 boundary, note that one codon, TTA, was retained at this site (a). (B) Part of the sequence of the abnormal variant observed in a case of ovarian carcinoma (in reverse direction). A 536 nucleotide sequence (b–c) was inserted at the exon 10–11 boundary.

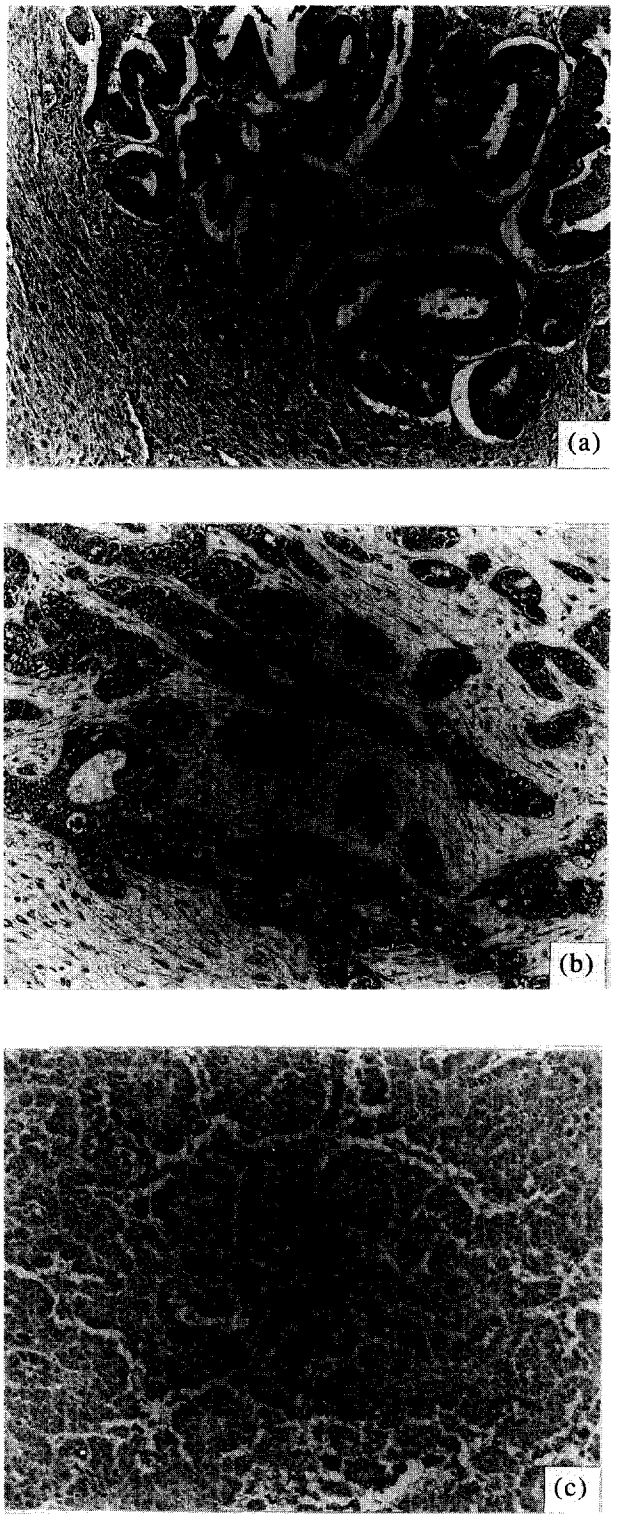

Figure 3. Immunohistochemical expression of LH/hCG receptor protein. (a) Ovarian carcinoma with both full-length and smaller fragments of LH/hCG mRNA by RT-nested PCR stained positive for the receptor protein. (b) Ovarian carcinoma with only the smaller fragment stained positive. (c) Ovarian carcinoma with no fragments of LH/hCG mRNA stained negative. Original magnification ×400.

and intracellular domains. The 536 nucleotide region showed no sequence homology to any part of the LH/hCG receptor cDNA sequence and possibly originated from intron 10, since the sequence of the first 9 nucleotides was the same as that of intron 10 of the rat LH/CG receptor gene [24].

LH/hCG receptor protein expression detected by immunohistochemistry

According to the LH/hCG mRNA splicing pattern, of the 7 cases of ovarian carcinoma examined by immunohistochemistry, 2 cases had both fragments, 1 had the smaller fragment only, and the other 4 had neither fragment. Positive immunostaining for LH/hCG receptor protein was observed in two cases which exhibited both the fragments (Figure 3(a)), and in one case with the exon 9-lacking variant (Figure 3(b)). In contrast, it was negative in the remaining four carcinomas in which no fragments could be amplified.

*Relationship between LH/hCG receptor expression and clinicopathological status, expression of EGFR and c-erb*B-2 *and patient survival in ovarian carcinoma*

The correlation between the expression of LH/hCG receptor detected by RT-nested PCR and the clinicopathological status was examined in 43 cases of ovarian carcinoma. LH/hCG receptor expression was defined as positive when the full-length variant was detected by RT-nested PCR, and 17 of the 43 (40%) carcinomas were positive for LH/hCG receptor. With regard to patient's age, LH/hCG receptor was positive in 10 of the 17 (59%) patients of age 50 years or more, and 9 of the 26 (35%) patients of less than 50 years of age ($P = 0.118$). With respect to the disease stage LH/hCG receptor positivity was detected in 4 of the 12 (33%) stage I, 1 of the 3 (33%) stage II, 10 of the 20 (50%) stage III, and in 2 of the 8 (25%) stage IV carcinomas (Table 1). There was no significant correlation between LH/hCG receptor expression and clinical stage of the disease. According to the histological subtype of carcinoma, LH/hCG receptor expression was detected in 10 of the 24 (42%) serous, 1 of the 2 (50%) mucinous and 6 of the 11 (55%) endometrioid carcinomas. None of the 5 clear cell carcinomas or one transitional cell carcinoma expressed the full-length LH/hCG receptor transcript, while approximately half of the other histological types did (0% versus 46%, $P = 0.033$).

The relationship between LH/hCG receptor positivity and the levels of EGFR and c-*erb*B-2 mRNAs detected by RT–PCR in 40 cases of ovarian carcinoma [24] were examined. The level of EGFR expression was slightly higher in LH/hCG receptor positive than receptor-negative carcinomas ($P = 0.10$), although there were no significant relationships between LH/hCG receptor expression and EGFR or c-*erb*B-2 expression (data not shown).

With regard to prognosis, the patients with LH/hCG receptor-positive carcinoma showed a significantly better survival rate compared with those without the receptors ($P = 0.047$) during the follow-up period of less than 5 years (Figure 4).

DISCUSSION

The present study demonstrates the expression of LH/hCG receptor mRNA and protein in normal and neoplastic human ovarian tissues. In all the normal ovarian tissues examined, RT-nested PCR amplified two different size fragments about the same size as previously reported [13]. Subcloning and sequencing revealed that the larger frag-

Table 1. Incidence of LH/hCG receptor mRNA variants in normal ovary and various ovarian tumours

Histological diagnosis	Total number of cases	L(+)/S(−)	L(−)/S(+)	L(−)/S(−)
Normal ovary	5	5 (100)	0 (0)	0 (0)
Epithelial ovarian tumours	55	26 (47)	7 (13)	22 (40)
benign cystadenoma	5	4 (80)	1 (20)	0 (0)
borderline tumour	7	5 (71)	2 (29)	0 (0)
Carcinoma stage	43	17 (40)	4 (9)	*22 (51)
stage I	12	4	1	7
stage II	3	1	1	1
stage III	20	10	2	*8
stage IV	8	2	0	6
Histology				
serous	24	10	2	*12
mucinous	2	1	0	1
endometrioid	11	6	1	4
clear cell	5	0	1	4
transitional	1	0	0	1
Sex cord–stromal tumors	3	3 (100)	0 (0)	0 (0)
granulosa cell tumour	2	2	0	0
thecoma	1	1	0	0
Germ cell tumours	4	0 (0)	0 (0)	4 (100)
dysgerminoma	3	0	0	3
yolk sac tumour	1	0	0	1
Ovarian cancer cell line	2	1 (50)	0 (0)	1 (50)

L, larger fragment of LH/hCG receptor mRNA; S, smaller fragment of LH/hCG receptor mRNA. *Including one case with an unusually large fragment of LH/hCG receptor mRNA. ( ), percentage.

ment was derived from a full-length LH/hCG receptor cDNA [5], whereas the smaller fragment represented a truncated form in which exon 9 was spliced out. In our study, however, the splicing pattern of the smaller transcript was slightly different from that described in a previous report [5]; three bases, TTA, which were reported to be included in the spliced-out portion, were retained at the exon 8–10 boundary, resulting in retention of a leucine residue in the protein product. It has recently been recognized that alternative splicing of pre-mRNA is a widespread mechanism in the regulation of gene expression [25]. In rats, many variant forms of LH/CG receptor have been reported to be produced by alternative splicing and polyadenylation [26]. In human corpora lutea, various sizes of mRNA have also been detected by Northern blot analysis [9]. The alternative splicing of the primary transcript of LH/hCG receptor appears to be different among species and organs [7, 27], and has been suggested to be regulated by some yet unknown mechanism [8, 28, 29]. The truncated N-terminal half of the receptor was shown to be capable of high-affinity ligand binding without cAMP production [30].

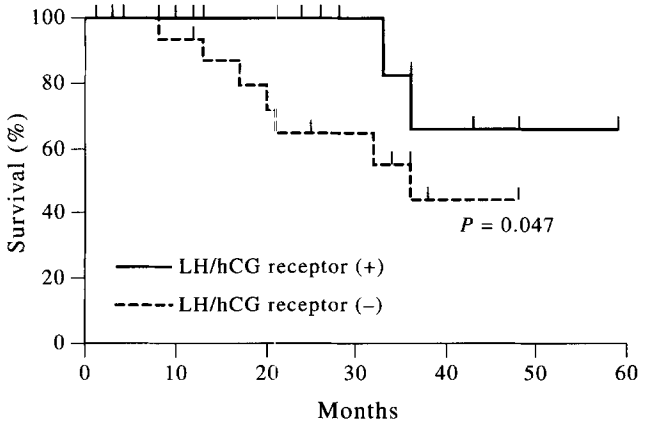

Figure 4. Survival curves of patients with or without LH/hCG receptors in ovarian carcinomas. The curves were generated by the Kaplan–Meier method, and significant differences were calculated by the generalised Wilcoxon test.

Our study has shown that ovarian carcinomas also express a full-length variant mRNA of LH/hCG receptor and its splice variant; 17 of the 43 carcinomas, 5 of the 7 borderline tumours and 4 of the 5 benign tumours expressed both fragments, while 4 carcinomas, 2 borderline tumours and 1 benign tumour expressed only a small matter fragment lacking exon 9. In addition, one case of stage III ovarian carcinoma expressed an unusually large size fragment possibly yielding a truncated LH/hCG receptor protein. In malignant neoplasms, splicing variation in specific genes may play a role in tumour development or progression [31–33]. Further investigations are needed to elucidate whether the splicing pattern of the LH/hCG receptor gene associated with the development or progression of epithelial ovarian neoplasms.

The possible relationship between gonadotropins and ovarian cancer is currently the subject of ongoing debate [16, 34–42], since Whittemore and associates reported a relative risk of 2.8 for ovarian carcinoma in women who had used fertility drugs [34]. Expression of the receptor for another gonadotropin, follicle-stimulating hormone (FSH), has recently been reported in normal ovarian surface epithelium that is regarded as an origin of ovarian epithelial tumours [43]. LH/hCG receptor mRNA of both the full-length and its splicing variant is also expressed in cultured ovarian surface epithelial cells (unpublished data). There have been case reports of ovarian and peritoneal cancers in infertile women during or after ovulation-induction therapy with gonadotropins or other drugs [35, 44–56]. Apparently rapid growth of ovarian cancer has been observed during early pregnancy when the serum hCG level was markedly elevated [51, 56]. However, with regard to receptiveness of tumour cells to gonadotropins in ovarian carcinomas, there

are conflicting data based on the binding assays for FSH or LH/hCG [17–20]. The present study has demonstrated that full-length LH/hCG receptor mRNA was expressed in 40% of ovarian carcinomas, 71% of borderline tumours and one of the two ovarian cancer cell lines. Immunohistochemical analysis has also confirmed the localisation of the receptor protein in the tumour cells. Increased tumour cell growth *in vitro* by gonadotropin, either FSH or LH/hCG, has been demonstrated in human ovarian cancer cells [57–60]. These findings suggest that the tumour cells of ovarian epithelial carcinomas do express LH/hCG receptors.

In our series, the expression of full-length LH/hCG receptor mRNA in tumour tissues was more frequently detected in women aged 50 years or more, although the difference was not statistically significant. As regards the histological subtype, the LH/hCG receptor mRNA was negative in all 5 clear cell carcinomas, but was positive in approximately half the other histological types. Patients with LH/hCG receptor-positive carcinomas showed a better survival rate compared with those that did not express the receptors. These findings suggest that ovarian carcinomas with positive LH/hCG receptor expression may represent a hormone-dependent, less-aggressive phenotype. Recently, the modulation of several oncogene products or growth factors such as EGF and IGF-I by gonadotropins has been reported in ovarian follicular cells [30, 61]. There was no significant correlation between the LH/hCG receptor expression and levels of EGFR or c-*erb*B-2 mRNAs in our study. Further analysis is needed to clarify the relationship between LH/hCG receptor expression and oncogene/growth factors in ovarian carcinomas.

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