Abstract. Background: Recent studies proposed L1CAM (L1 cell adhesion molecule) expression as a negative prognostic marker in epithelial ovarian cancer (EOC). The gene L1C was screened for single nucleotide polymorphisms (SNPs) which could impact upon EOC risk or disease progression. Patients and Methods: Overlapping DNA fragments, including the promoter region, intron 1 and all the exons of 10 healthy volunteers were analyzed to detect SNPs. EOC patients (n=103) and age-matched controls (n=104) were subsequently genotyped by restriction fragment length polymorphism (RFLP). Quantitative real-time PCR was carried out to detect potential associations of SNPs with L1C mRNA expression. Results: One SNP was found in intron 1 (L1C G842A). Genotyping of the EOC patients and age-matched controls revealed an association of EOC with the homozygous AA genotype (OR 7.4, CI 1.6-33.5; p=0.003). The L1C mRNA expression levels and clinical parameters did not differ significantly between the L1C G842A genotypes. Conclusion: The L1C 842 AA genotype may be a predisposing factor for EOC.

L1CAM (L1 cell adhesion molecule) is a transmembrane Ig-like glycoprotein of 200-220 kDa which was initially described in neuronal tissues. It is involved in heterophilic and homophilic interactions and its cytoplasmic tail interacts with the cytoskeleton via ancrin binding sites (1, 2). Mutations of L1C are associated with nonsyndromal X-chromosomal inherited hydrocephalus and other neurological disorders (3, 4). L1CAM appears to be a dynamic receptor with multiple binding mechanisms and functions (1), it exerts properties of a signal transduction receptor as well as an adhesion molecule (5-7). L1CAM is involved in the induction of extracellular regulated kinase (ERK)-dependent gene regulation (8). Amongst others, TAX1 (human T-cell leukemia virus type I) and F11 (contactin) have been identified as ligands for L1CAM in axonal growth (4,9). L1CAM is involved in the regulation of angiogenesis through interactions with αvβ3 (αvβ3-integrin) (10).

Recently, functions of L1CAM have become of greater interest, as the expression of this molecule has been found to correlate with poor prognosis in glioblastomas, melanomas, endometrial cancer and ovarian cancer to name but a few (11-16). A correlation of L1CAM expression with stage, grading and progression-free survival in serous ovarian neoplasms has been described recently (17). An important pathophysiological mechanism of L1CAM in tumours seems to be the induction of gene products associated with cell motility and invasion such as β3-integrin and cathepsin B via the ERK-dependent pathway (8). A soluble form of L1CAM has been found in the sera and ascites of ovarian cancer patients, also indicating a role in tumour biology (18). In transfection models, L1CAM caused diminished apoptosis in ovarian cancer cell lines (19).

L1CAM is also a target for antibody therapies and promising results have already been reported both in vitro and in vivo (14, 20, 21).

The L1C gene is located on the X-chromosome and consists of 28 exons. Exon 2 and 27 are spliced out in an isoform of L1CAM which is expressed exclusively in non-neuronal tissue (22).

Due to the apparent prognostic role of L1CAM, especially in ovarian tumours, the L1C gene was screened for single nucleotide polymorphisms (SNPs) which could impact upon the clinical course of disease in ovarian cancer patients.

Patients and Methods

Patients/Volunteers. One hundred and three patients who had been treated between 1999 and 2007 for ovarian cancer in the University Hospital of Essen were enrolled in this study. The clinical data were documented in a database. Healthy, age-matched female blood donors...
Genotyping for L1C G842A.

The DNA samples from the healthy Kallunki nonneuronal isoform of L1CAM. Nearly 10 kb upstream of exon 1, special regard to the splice sites, as exon 2 is spliced out in the start codon ATG, all the exons and intron 1. Intron 1 was included with PCR products for the promoter region up to –1192 bp upstream of the reference sequences, primer pairs were designed to amplify overlapping volunteers, 8 females and 2 males, were analysed. Using available SNPs, the PCR products derived from the DNA of 10 healthy unrelated volunteers, (DNASTAR Inc., Madison, WI, USA) for Windows©.

In order to identify Detection of single nucleotide polymorphisms.

The local Ethics Committee. Patients with borderline tumours or germ cell tumours were enrolled including serous, mucinous, clear cell and endometrioid grading was conducted using the grading system proposed by the WHO classification of tumours of the female genital tract (23), classified as FIGO (Fédération Internationale de Gynécologie et d’Obstétrique) III or IV, thus indicating a representative collective of ovarian cancer patients (Table I). The patients’ mean age at primary diagnosis was 57 years, 80 (77.6%) of the tumours were classified as FIGO (Fédération Internationale de Gynécologie et d’Obstétrique) III or IV, thus indicating a representative collective of ovarian cancer patients (Table I). The median follow-up time was 24 months (1-131 months). The diagnosis was confirmed by histopathology in all cases. The tumours were classified according to the WHO classification of tumours of the female genital tract (23), grading was conducted using the grading system proposed by Silverberg (24). Patients with epithelial ovarian neoplasms were enrolled including serous, mucinous, clear cell and endometrioid histology. Patients with borderline tumours or germ cell tumours were excluded from the analysis. Approval for this study was obtained from the local Ethics Committee.

**Detection of single nucleotide polymorphisms.** In order to identify SNPs, the PCR products derived from the DNA of 10 healthy unrelated volunteers, 8 females and 2 males, were analysed. Using available reference sequences, primer pairs were designed to amplify overlapping PCR products for the promoter region up to –192 bp upstream of the start codon ATG, all the exons and intron 1. Intron 1 was included with special regard to the splice sites, as exon 2 is spliced out in the nonneuronal isoform of L1CAM. Nearly 10 kb upstream of exon 1, Kallunki et al. described an additional exon in the 5’ flanking sequence (25). Consequently, this region was also included in the present analysis. DNA was extracted from whole blood or paraffin embedded tissue using a QIAamp kit (Qiagen, Hilden, Germany). DNA sequencing was performed by an external service (Eurofins Medigenomix, Martinsried, Germany). Reference sequences and sequenced fragments were analysed using DNASTAR MegAlign© (DNASTAR Inc., Madison, WI, USA) for Windows©.

**Genotyping for L1C G842A.** The DNA samples from the healthy voluntary blood donors and ovarian cancer patients were retrospectively genotyped for the L1C G842A polymorphism. PCR was performed with the forward primer 5’-TTAACATGGATACACTGTGTA-3’ and the reverse primer 5’-CAAATGTGGTGGTCAAATAA-3’, resulting in a 165 bp fragment. After denaturation at 95°C, 38 cycles of DNA amplification were conducted using Taq PCR Mastermix (Eppendorf, Hamburg, Germany) at 95°C for 40 s, 56 °C for 40 s, and 72°C for 40 s. Digestion with the restriction endonuclease HphI (New England Biolabs Inc., Ipswich, MA, USA) resulted in fragments of 102 bp and 63 bp for the G allele vs. 165 bp for the A allele (no digestion). Electrophoresis was performed in 2.5% agarose gels using SYBR Safe® DNA Gel Stain (Invitrogen Corporation, Carlsbad, CA, USA) for visualization under UV light.

**Real-time PCR.** The total RNA was isolated and purified from 19 ovarian cancer specimens using a RNeasy Mini-Kit® (Qiagen). After extraction, reverse transcription to cDNA was conducted using the SuperScript II® System (Invitrogen Corporation) according to the manufacturers instructions. L1C mRNA expression was determined by RT-PCR using L1C cDNA specific primers. SYBR Green® (Qiagen) chemistry was used to detect the primer specific amplicons. The relative gene expression levels were calculated in relation to the corresponding beta-actin gene expression levels.

**Statistical analysis.** Deviations from Hardy-Weinberg equilibrium (HWE) were tested using the Chi-square test according to Pearson. The genotype frequencies in the patients and controls were compared using the Chi-square test. Kaplan-Meier survival analysis was applied to investigate the prognostic importance of the genotypes concerning overall and progression-free survival. Progression-free survival was calculated from the date of initial diagnosis to the time of diagnosed progressive disease. Overall survival was calculated from the time of initial diagnosis to confirmed death from ovarian cancer. Comparison of the clinical and laboratory parameters between the patient subgroups was performed using the Kruskal-Wallis test for the continuous variables and the Chi-square test for the categorical data. Differences were regarded as statistically significant at $p<0.05$. Statistical analyses were performed using SPSS 15.0 for Windows (SPSS Inc., Chicago, IL, USA).

**Results**

Upon sequencing more than 10 kb of DNA from 10 individuals, one SNP in intron 1 was found. The polymorphism rs4646263 consisted of a base exchange from guanine to adenine and was located 842 bp downstream of ATG.

### Table I. Clinical data of ovarian cancer patients (n=103).

<table>
<thead>
<tr>
<th>Age at first diagnosis (years)</th>
<th>FIGO stage</th>
<th>Histopathology</th>
<th>Relapse or progressive disease</th>
<th>Platinum resistance</th>
<th>Progression-free survival (months)</th>
<th>Follow-up (months)</th>
</tr>
</thead>
<tbody>
<tr>
<td>57 (23-83)</td>
<td>I</td>
<td>Serous</td>
<td>43 (42%)</td>
<td>16 (16%)</td>
<td>18 (1-131)</td>
<td>24 (1-131)</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>Mucinous</td>
<td>53 (51%)</td>
<td>16 (16%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>III</td>
<td>Clear cell</td>
<td>3 (3%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>IV</td>
<td>Endometrioid</td>
<td>9 (9%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Other</td>
<td>10 (10%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Macrosopically tumour free resection in primary surgery</td>
<td>73 (71%)</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

### Table II. Genotype distribution of L1C G842A.

<table>
<thead>
<tr>
<th>G allele frequency</th>
<th>Ovarian cancer patients N=103</th>
<th>Age-matched controls N=104</th>
</tr>
</thead>
<tbody>
<tr>
<td>GG</td>
<td>52 (50%)</td>
<td>62 (60%)</td>
</tr>
<tr>
<td>GA</td>
<td>38 (37%)</td>
<td>40 (38%)</td>
</tr>
<tr>
<td>AA</td>
<td>13 (13%)</td>
<td>2 (2%)</td>
</tr>
</tbody>
</table>

$p=0.011$
The genotype distributions in the patients and controls are shown in Table II, they were compatible with Hardy Weinberg equilibrium for the patients as well as for the controls. Chi-square test revealed a statistically significant difference in the distribution of genotypes between the ovarian cancer patients and the controls (p=0.011). Interestingly, the AA genotypes were significantly more frequent in the patients with ovarian cancer. For the homozygous A allele carriers versus the GG genotypes, a significantly increased OR of 7.8 (95% CI: 1.7-35.9; p=0.005) was calculated. The OR for AA versus AG genotypes was calculated to be 6.8 (95% CI: 1.4-32.4; p=0.007), and an OR of 7.4 (95% CI: 1.6-33.5; p=0.003) was calculated for AA versus combined AG plus GG genotypes.

Regarding the clinical data such as age at primary diagnosis, histopathological tumour type, grading, staging according to FIGO and response to platinum-based chemotherapy, no statistically significant differences were seen between the different genotypes (data not shown). Kaplan-Meier analysis for progression-free survival and overall survival did not show divergent survival between the analysed genotypes.

Real-time PCR did not reveal genotype-dependent alterations in the LIC mRNA expression levels in the ovarian cancer tissue specimens.

Discussion

To the best of our knowledge, this was the first investigation of genetic alterations of LIC in tumour patients. In neuronal tissues, mutations of LIC have a substantial impact (3,4). During recent years LICAM was initially a focus of neuronal research, but subsequently emerged as a potential target molecule for antibody therapies (14, 20, 21). LICAM expression appears to have prognostic relevance in malignancies (11-16), recent findings have also indicated an involvement in molecular mechanisms leading to chemoresistance (26). The exact role of this molecule in neoplasms still remains to be elucidated.

A striking finding of the present work was that the sequence of LIC appears to be highly conserved. Admittedly, this conclusion is based upon the analysis of ten healthy volunteers only and it could be argued that more probands might have revealed more polymorphisms. However, the present focus was to identify polymorphisms of considerable frequency with accordingly high clinical importance. Only one SNP was found, located in intron 1, which was the subject of the further investigations. As previously reported by others, SNPs in the first intron can be of functional importance due to alteration of transcriptional activity (27, 28) and are, therefore, of potential relevance.

The findings indicated an association of the LIC 842 AA genotype with an increased risk for ovarian cancer, while the course of the disease was not associated with the LIC G842A genotypes. While the association between the LIC 842 AA genotype and ovarian cancer appeared strong, the underlying mechanism remains unclear. Since the mRNA expression levels did not diverge between genotypes, it can be hypothesized that the evaluated SNP is not the causative genetic factor. Probably, other polymorphisms which are in linkage disequilibrium and might be located in adjacent chromosomal regions account for the observed association.

Confirmation of these results in a larger, independent patient collective is mandatory. In future, it will be interesting to evaluate the impact of this polymorphism on an increased risk for other malignancies.

Conflict of Interest

This manuscript has been approved by all authors. There are no relationships that may present conflicts of interest.

References


