Associations of CXCL1 gene 5’UTR variations with ovarian cancer

CURRENT STATUS: UNDER REVISION

Journal of Ovarian Research  BMC

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DOI:
10.21203/rs.2.18650/v3
SUBJECT AREAS
- Cancer Biology
- Sexual & Reproductive Medicine

KEYWORDS
- Ovarian cancer; Chronic inflammation; Chemokines; CXCL1; 5’UTR
Abstract
Background: There are about 2.4 hundred thousand new cases and 1.5 hundred thousand deaths of ovarian cancer (OC) annually in the world. Chronic inflammation is a risk factor for OC. C-X-C motif chemokine ligand 1 (CXCL1) defects may facilitate inflammation and transactivate EGFR in ovarian cancer, but the precise haplotypes associated with the potential diseases remained largely unknown. In this work, we characterized CXCL1 gene variations to elucidate their possible associations with OC.
Methods: We analyzed the CXCL1 gene for 300 OC patients with 400 healthy participants as controls. The statistical analyses and Hardy-Weinberg equilibrium tests of the patients and control populations were conducted using the SPSS software (version 19.0) and Plink (version 1.9). Results: The variants rs11547681, rs201090116, rs199791199, rs181868085, rs4074 and rs1814092 within or near the CXCL1 gene were characterized. The genetic heterozygosity of rs11547681 and rs4074 was very high. Statistical analysis showed that the variant rs11547681 in the gene was closely associated with the risk of OC in the Chinese Han population, although this variant was not associated with FIGO stages or pathological grades of the patients. Conclusions: Rs11547681 in CXCL1 gene was associated with the risk of OC in the Chinese Han population.

Introduction
In the world, there are about 2.4 hundred thousand new ovarian cancer (OC) cases and about 1.5 hundred thousand deaths caused by this disease are reported annually [1]. OC is the 7th most common and 5th most lethal cancer among women worldwide [2]. The incidence rates of OC are highest in northern and eastern Europe, and lowest in Asia and Africa [1]. However, although the rates have decreased in many high incidence countries in the past decades, the morbidity has been increasing in many previously low incidence countries, such as China [1, 3]. The 5-years survival rates of OC range from 30.3% to 44.1%, depending on the specific subtypes and stages at the time of diagnosis or treatment [4, 5]. Early detection and treatment of this disease can increase the survival rate of OC. However, without specific signs or symptoms at early stages, this disease can usually be diagnosed only at late stages [6]. The recurrence rate of this disease is also very high, often leading to death of the patient, who might have achieved a clinical complete
remission after primary therapy [5, 6].

So far, several genetic and non-genetic factors have been found to be associated with OC. Women with affected first-degree relatives usually have higher risks for OC [7], and women, who have relatives diagnosed with OC below 50 years old, have even higher OC risks [8]. Many gene mutations have been associated with sporadic OC patients, including BRCA1 and 2 [9], BRIP1 [10] and RAD51 [11]. Some non-genetic factors include hormonal levels, oral contraceptive use and tubal ligation seem to be protective factors [12, 13], whereas older menopausal ages, obesity, menopausal hormone therapy use, a history of endometriosis and smoking are all risk factors [14-16]. Chronic inflammation, such as those caused by asbestos or talc exposure, endometriosis or pelvic inflammatory diseases, has also been suggested to be a risk factor for OC [17, 18]. On the other side, anti-inflammatory medications, e.g., acetaminophen and low-dose of aspirin are protective factors for OC [19, 20]. Immunotherapies, including immune checkpoint blockade and cancer vaccines, also have many special roles in immune recognition and immune regulation of the OC cells [21].

The chemokines are expressed in tumor or stromal cells, can increase tumor angiogenesis and suppress immune-mediated tumor elimination, and are associated with many human diseases including cancers, autoimmune and inflammatory diseases [22-24]. C-X-C motif chemokine ligand 1 (CXCL1) is a member of the CXC subfamily of chemokines and an oncogenic factor in many cancers [25, 26]. It can transactivate EGFR in OC through binding to the G-protein coupled receptor CXC receptor 2 (CXCR2) [27]. Abnormal expression of CXCL1 is associated with many tumors [28], but the associated haplotypes remained largely unknown.

In this work, we investigated variants in the CXCL1 gene for their associations with the risk of OC in the Chinese Han population. We found that variant rs11547681 was associated with ovarian cancer and demonstrated the 5’UTR for the functions of CXCL1.

Materials And Methods

Study population

A total of 300 sporadic OC cases and 400 normal controls (Table 1) were assembled for this study at the Department of Gynaecology and Obstetrics and Medical Examination Center of the Second
Affiliated Hospital of Harbin Medical University, Harbin, China. From each participant, we obtained a written informed consent. This work has been reviewed and approved by the Ethics Committee of Harbin Medical University. We also confirmed that all experiments were performed in accordance with relevant guidelines and regulations. All those were consistent with the 1975 Declaration of Helsinki. Medical histories of the enrolled participants were recorded in detail, and all the participants were received physical examinations. The diagnostic criteria for sporadic OC patients were FIGO (Federation International of Gynecology and Obstetrics) and those patients had no history of other systemic abnormalities or previous tumor or familial history of tumor. The exclusion criteria for the control participants were not had any disease or systemic abnormalities.

**DNA analysis**

We used standard protocols to extract the genomic DNA from each participant peripheral blood leukocytes as described previously [29]. The CXCL1 gene was amplified by PCR with the primers (Table 2). PCR products were sequenced using standard protocols [30, 31] for genotype analysis.

**SNP genotyping and Statistical analysis**

The variations within or near the CXCL1 gene were determined for the 300 sporadic OC cases and 400 normal controls. The DNA regions were amplified and the PCR products were sequenced to determine the genotypes; 2 researchers conducted the measurements independently. Overall OC genetics correlation analysis was also conducted. As previously reported, the statistical analyses and Hardy-Weinberg equilibrium tests of the patients and control populations were conducted [29, 32-34].

**Results**

**Clinical data**

The clinical diagnosis of all the participants was confirmed by specialists in Department of Gynaecology and Obstetrics in the Second Affiliated Hospital of Harbin Medical University, Harbin, China. These OC patients had no history of other systemic abnormalities or previous tumor or familial history of tumor. All the OC patients (n = 300, female, average age was 50.39 years, the min and max age were 18 and 81 respectively) and normal controls (n = 400, female, the average age was 49.68, the min and max age were 31 and 67 respectively) were recruited specifically for this study. There
were no statistical differences in age composition between the two groups (Table 1).

**SNP gene analyses**

In order to test the hypothesis that germline common genetic variants in *CXCL1* gene may be associated with the susceptibility to OC, we extracted the genomic DNA from the peripheral blood leukocytes of all the participants and sequenced the *CXCL1* gene to detect SNPs. We found six SNPs distributed on the gene, including rs11547681, rs201090116, rs199791199, rs181868085, rs4074 and rs1814092 (Fig 1A). Analysis of these SNPs showed that the genetic heterozygosity of rs11547681 and rs4074 was very high (Fig 1B), whereas that of rs201090116, rs199791199, rs181868085 and rs1814092 was very low and were excluded from further analysis.

**Polymorphism-disease association analyses**

To test the hypothesized associations between *CXCL1* variations and OC, we conducted analyses on the SNPs and found the variant rs11547681 within the 5′UTR of the gene was associated with the risk of OC in the Chinese Han population (Tables 3 and 4). Further, we analyzed the Hardy–Weinberg equilibrium test for the study population groups, and it was in line with equilibrium (Table 5). The genotype frequencies in the two groups were also analyzed by three genetic models (trend, dominant and recessive), and we found that the rs11547681 was associated with the risk of OC in trend and dominant models (Table 6). For the variant rs4074, we did not found statistical significance between the OC and control groups (chi-square tests; trend, dominant and recessive models). We also want to compare the genotype frequency of the rs11547681 and rs4074 in the two groups and the data from the HapMap HCB population, however we could not find the genotype frequency data of rs11547681 in the HapMap HCB population.

**Clinical features comparative analysis**

We also compared the clinical characteristics between the wild type, heterozygous variant and homozygous variant groups of the OC patients. We found there were no statistically differences between the three groups in FIGO stages and pathological grades (Table 7).

**Discussion**

In this study, we found that the SNP rs11547681 within the 5′UTR of *CXCL1* gene was associated with
OC. The microenvironment of tumors is an important factor in modulating cancer development, especially in organs that communicates with the outside world [35, 36]. The cells surrounding those tumors usually release some factors, such as growth or inflammatory factors, which regulates inflammation or progression of tumors [37, 38].

The human chemokines have strong activity in tumor cells, especially in cross talk of tumor cells and their host microenvironment [39]. CXCL1 is one member of the chemokines, which is a proinflammatory mediator in many inflammatory diseases. CXCL1 promotes and exacerbates growth and progression of many tumors [40]. By activating CXCR2, CXCL1 is associated with cancer cell growth and proliferation, tumor angiogenesis and metastasis [41, 42]. In tumor therapies, CXCL1 is also responsible for several chemotherapeutic drugs resistance [43].

In the OC cells, over-expression of CXCL1 factor promotes the abilities of cellular proliferation and invasion in vitro [27, 44]. Progesterone and calcitriol can inhibit ovarian and endometrial cancer cell growth by attenuation functions of CXCL1, and if the expression of CXCL1 is reduced, the inhibitory effect of the two agents is also abrogated [45]. In the other side, when the expression of CXCL1 is increased, the activation of metastasis promoting gene p65 is also increased in OC cells [45]. The serum CXCL1 is also may be a novel tumor marker for OC diagnosis [46]. In this study, we found the SNP rs11547681 in the CXCL1 gene was associated with OC. Further enhances the special roles of CXCL1 factor for the pathogenesis, diagnosis and therapies of ovarian cancer.

Chemokines is exists as monomers and dimers in vivo. Its function is produced by binding to tissue glycosaminoglycans (GAGs) heparan sulfate (HS), chondroitin sulfate (CS) and dermatan sulfate (DS) [47, 48]. GAGs is binding to a diversity of protein classes [49], so in order to interact with GAGs, the sequence of chemokine must play special roles in determining selectivity, affinity and geometry [50]. CXCL1 belong to the CXC chemokines subset, characterized by the N-terminal‘ELR’motifs [51]. The amino acid residues located within the N terminal loop and C terminal helix of CXCL1 factor mediates HS binding, the participation of other residues may results in a very different binding geometry for CXCL1 [50]. The SNP rs11547681, we found was associated with OC, is located within the 5‘UTR of CXCL1 gene. The 5‘UTR and 3‘UTR sequences are regulates expression of genes [52, 53]. The 5‘UTR
sequences of gene is binding with miRNAs, may be involved in gene expression, protein translation or
disease pathogenesis [54]. In previous study, we found SNPs within the 5′UTR or 3′UTR sequences are
associated with diseases [31, 32, 55]. So, the results of this work further emphasized the important
roles of 5′UTR sequences for CXCL1 factor functions.

In conclusion, we validated the associations of CXCL1 variants rs11547681 with the risk of ovarian
cancer in the Chinese Han population, and updated our understanding on 5′UTR for CXCL1 functions.
Those may lead to new insights into the pathogenesis of cancers especially ovarian cancer.

Declarations

Author Contributions: Conceptualization: FF L, SL L; methodology: FF L, SL L, M G, C X; software: FF
L, SL L, C X; formal analysis: FF L, SL L, M G, C X; investigation: M G, C X, YZ C, QW S, Y Y, YH H;
resources: M G; data curation: FF L, SL L, M G, C X; writing: FF L, SL L; funding acquisition: FF L, SL L

Acknowledgements: The authors thank the patients and their families for their cooperation and
participation in this study.

Competing Interests: All the authors have declared that no competing interests exist.

Financial disclosures: There are no financial disclosures from any authors.

Availability of data and materials: The datasets used in the present study are available from the
corresponding authors with reasonable requests.

Consent for publication: Not applicable.

Funding Statement: This work was supported by grants from Health and Family Planning
Commission of Heilongjiang province Foundation (2017-077), Postdoctoral Foundation of
Heilongjiang Province and grants of National Natural Science Foundation of China (NSFC81271786,
81030029, 81671980), Heilongjiang Innovation Research Foundation for College Students
(201810226070). The funders had no role in study design, data collection and analysis, decision to
publish, or preparation of the manuscript.

Ethics approval: Ethics Committee of Harbin Medical University.

Informed consent: Informed consent was obtained from all individual participants.

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### Tables

#### Table 1: Clinical characteristics of study population.

| Parameter | CRC | Control | F  | t  | P     |
|-----------|-----|---------|----|----|-------|
| Sample (n) | 300 | 400     | -  | -  | -     |
| Age (years) | 50.39±12.21 | 49.68±7.66 | 9.977 | 0.639 | 0.523 |

Data are shown as mean±SD; between the two groups, there were no statistical differences of the age and gender composition.

#### Table 2: PCR primers used for CXCL1 gene sequence analysis

| Exon     | Forward primer          | Reverse primer          |
|----------|-------------------------|-------------------------|
| Exon1    | GCGGGCTGCATCAGTGGGA     | CCGGACTTACATGACTTCGGT   |
| Exon2    | CTGCTGCTCCTGCTCCTGGTA   | GGAAAGGGAATCTCGTGAGGC   |
| Exon3    | AAACCGAAGTCATGTAAGTCC   | CAATAATCCCAATTTCATGC    |
| Exon4a   | TTAGAGGTCCTGCCCTCCACA   | ATTTCCCCTGCCTTCACCC     |
| Exon4b   | TGCAACATGCGCCAGCCT     | ATAGCAAATTGAACACCC      |
Table 3: The genotype and allele frequency of variations in 300 Chinese Han ovarian cancer patients and 400 normal controls.

| SNP          | Group | Genotype frequency (%) | Allele frequency (%) |
|--------------|-------|------------------------|----------------------|
| rs11547681   |       |                        |                      |
|              | Genotype | G/G | G/T | T/T | G   |
| OC           | 300    | 185 (61.7) | 107 (35.7) | 8 (2.7) | 477 (79.5) |
| Controls     | 400    | 294 (73.5) | 94 (23.5)  | 12 (3.0) | 682 (85.5) |
| rs4074       |       |                        |                      |
|              | Genotype | G/G | G/A | A/A | G   |
| OC           | 300    | 84 (28.0)  | 153 (51.0) | 63 (21.0) | 321 (53.5) |
| Controls     | 400    | 120 (30.0) | 207 (51.8) | 73 (18.3) | 447 (55.5) |

Note: OC = Ovarian Cancer

Table 4: rs11547681 variations within 5’UTR of the CXCL1 gene associated with risk of ovarian cancer in Chinese populations

| Variations | Type      | Pearson Chi-square | Risk                  |
|------------|-----------|---------------------|-----------------------|
|            | Value     | Min count | df | Asymp. Sig. (2-sided) | Value | 95% CI-low | 95% CI-up |
| rs11547681 | Genotype  | 12.412     | 8.57 | 2     | 0.002* | -        | -        |
|            | Allele    | 7.954      | 103.29 | 1 | 0.005* | 0.671 | 0.508 | 0.886 |
| rs4074     | Genotype  | 0.921      | 58.29 | 2 | 0.631 | -      | -        |
|            | Allele    | 0.781      | 270.86 | 1 | 0.377 | 0.909 | 0.735 | 1.124 |

*: statistically significant

Table 5: Hardy-Weinberg equilibrium test for the study population groups
**Table 6:** SNP rs11547681 within CXCL1 gene associated with the risk of ovarian cancer

| SNPs       | Value | Trend model | Dominant model | Recessive model |
|------------|-------|-------------|----------------|-----------------|
| rs11547681 | ChisQ | 8.0140      | 11.1100        | 0.0686          |
|            | P     | 0.0046*     | 0.0009*        | 0.7933          |
| rs4074     | ChisQ | 0.8121      | 0.3321         | 0.8282          |
|            | P     | 0.3675      | 0.5644         | 0.3628          |

*: statistically significant

**Table 7:** Comparative analysis of clinical features between wild type, heterozygous variation and homozygous variation groups

| Clinical Index                        | Wild Type       | heterozygous variation | homozygous variation |
|---------------------------------------|-----------------|------------------------|----------------------|
| TNM Stage (I/II/III/IV)               | 59/32/92/2      | 30/23/54/1             | 2/1/5/0              |
| Pathological Grades(H/M/L/Non)        | 101/15/36/33    | 54/7/31/15             | 5/0/3/0              |

H: Pathological high Grade; M: Pathological moderately Grade; L: Pathological low Grade;
Non: No pathological grade.

Figures
Figure 1

Schematic diagrams and DNA sequence chromatogram of SNPs in CXCL1 gene. A: locations of rs11547681, rs201090116, rs199791199, rs181868085, rs4074 and rs1814092 within the CXCL1 gene; B: DNA sequence chromatogram of the three polymorphisms identified in the CXCL1 gene in all the population used for disease-association analyses.