Author's response to reviews

Title: ICOS gene polymorphisms are associated with sporadic breast cancer: a case-control study

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Version: 3 Date: 6 August 2011

Author's response to reviews: see over
August 6, 2011

Dear Editor,

We are very pleased to learn from your letter about revision for my manuscript entitled “ICOS gene polymorphisms are associated with sporadic breast cancer: a case-control study” (MS: 1485703564560950). Thank you for your attention and the reviewers for their helpful comments and advice. We have revised the manuscript according to the comments from you and the reviewers. We provided the gel pictures of five SNPs in the additional file 1 as a supplemental figure. The revised manuscript had new line and page numbers, some grammar and spelling errors had also been corrected. The major revised portions were marked in blue for easy check/editing. We also responded point by point to each reviewer comments as listed below.

Reviewer's report

Title: ICOS gene polymorphisms are associated with sporadic breast cancer: a case-control study

Version: 2 Date: 30 June 2011

Reviewer: Oliver Treeck

Reviewer #1's report

Title: ICOS gene polymorphisms are associated with sporadic breast cancer: a case-control study

Version: 2 Date: 30 June 2011

Reviewer: Oliver Treeck

Reviewer's report:

Authors tested genotype and allele frequencies of ICOS SNPs in breast cancer patients and a control population. The question posed by the authors is well defined and the methods appropriate and well described. The data are sound and have been confirmed on a validation cohort, which is essential in SNP studies. Discussion and conclusions are well balanced and adequately supported by the data.

Level of interest: An article whose findings are important to those with closely related research
interests.

**Quality of written English:** Acceptable

**Statistical review:** No, the manuscript does not need to be seen by a statistician.

**Declaration of competing interests:**

I declare that I have no competing interests.

**Author responses**

Thank you very much for reviewing the manuscript. In order to make the article more understandable, we have made some changes to the manuscript.

**Reviewer #2's report**

**Title:** ICOS gene polymorphisms are associated with sporadic breast cancer: a case-control study

**Version:** 2  **Date:** 3 July 2011

**Reviewer:** Ke-Da Yu

**Reviewer's report:**

Major Compulsory Revisions

**Author responses**

Thank you very much for your attention and helpful comments and advice. We have revised the manuscript according to the comments. Here below is our description on revision.

**In Abstract**

1. The nomenclature of SNP is inconsistent. The five polymorphisms (rs11889031, IVS1+173, rs4675374, c.602 and c.1624) were addressed using either the RS number or the gene
location. The SNP such as IVS1+173 has a RS number in the NCBI db-SNP. Please revise the SNP name.

Thank you for your questions.

We have revised the SNP name for IVS1+173, c.602 and c.1624. The RS number for IVS1+173 is rs10932029, for c.602 is rs10183087 and for c.1624 is 10932037. The revised sentence is as below:

In the study cohort, we genotyped five SNPs (rs11889031, rs10932029, rs4675374, rs10183087 and rs10932037) in ICOS gene among 609 breast cancer patients and 665 age-matched healthy controls.

In addition, we have revised the SNP name for IVS1+173, c.602 and c.1624 in elsewhere in the revised manuscript (words in blue).

2. The difference between haplotype CTCAC and CCCAC is just due to the differential SNP of IVS1+173 (T and C). Does the haplotype analysis make sense?

Thank you for your questions.

In some studies, the single SNP was not associated with the risk of diseases, but there was an association between the reconstruction of haplotypes and risk of diseases, which suggest the importance of haplotype analyses (Ding J, et al: Association of PTEN Polymorphisms with Susceptibility to Hepatocellular Carcinoma in a Chinese Han Population. DNA Cell Biol 2011, 30(4):229-234), (Hadinia A, et al: CTLA-4 gene promoter and exon 1 polymorphisms in Iranian patients with gastric and colorectal cancers. J Gastroenterol Hepatol 2007, 22: 2283-2287). In our study, we conducted haplotype analyses to determine whether combinations of SNPs were associated with breast cancer risk. Haplotype CCCAC containing the rs10932029 (IVS1+173) C allele or haplotype CTCAC containing rs10932029 T allele was respectively
associated with higher or lower risk of breast cancer. Therefore, the rs10932029 SNP is likely to be driving these two haplotypes association, which highlight the important role for rs10932029.


In addition, it also indicated that only the combinations of rs11889031 C allele, rs10932029 T or C allele, rs4675374 C allele, rs10183087 (c.602) A allele and rs10932037 (c.1624) C allele were associated with the risk of breast cancer risk. The other haplotypes with different combinations of alleles showed no association.

Therefore, we do the haplotype analysis.

In order to illustrate this association better, we revised some sentences in the revised manuscript as below:

Abstract, results section: Haplotype analysis showed that CTCAC haplotype containing rs10932029 T allele had a lower frequency in cases than in controls (P=0.015), whereas haplotype CCCAC containing rs10932029 C allele was more common in cases than in controls (P=0.013).

Discussion section: We further analyzed the association between haplotypes and the risk of breast cancer. We found that CTCAC haplotype containing rs10932029 T allele appeared to be a protective against breast cancer, whereas CCCAC haplotype containing rs10932029 C allele may be a risk factor in breast cancer. This result also highlighted the important role for rs10932029.
2. The results have no data regarding survival. How the authors state that the “ICOS gene polymorphisms may affect the prognosis of breast cancer”. It is over-interpreted and the conclusion should be revised.

Thank you for your questions and advice.

The presence of hormone receptors such as estrogen receptor (ER) and progesterone receptor (PR) predicts the long-term outcome of hormonal therapy. ER-positive patients have a considerably better prognosis than ER-negative patients when patients treated with endocrine therapy. The two statuses have been more commonly used as predictive markers of endocrine therapy. Patients with CerbB-2 positive are at high risk for disease recurrence and cancer-related death. Moreover, CerbB-2 expression has been valuable in predicting treatment responses to trastuzumab, certain endocrine therapies and chemotherapy, adding to its role as a predictive marker. In our study, we found associations of prognostic factors (status of ER and PR) with certain genotypes and haplotypes. Additionally, we also observed that rs11889031 was associated with lymph node involvement. We thought that these SNPs can indirectly reflect the prognosis of breast cancer. Therefore, we summarized them that ICOS gene polymorphisms were associated with prognosis of breast cancer. However, we did not check the association between genotypes and survival of breast cancer patients. Carefully considering your questions above, we agreed that the conclusion “ICOS gene polymorphisms may affect the prognosis of breast cancer” was not proper here. To describe this relationship well, we have made some changes in our revised manuscript as below:

Abstract section: These results indicate that ICOS gene polymorphisms may affect the risk of breast cancer and show that some SNPs were associated with breast cancer characteristics in a northern Chinese population.
Background section: To determine the key roles of ICOS in tumor immunity, we genotyped five potentially functional SNPs, including rs11889031, rs10932029 (IVS1+173), rs4675374, rs10183087 (c.602) and 10932037 (c.1624), and investigate their associations with both the risk and clinicopathologic features of breast cancer in Chinese women from Heilongjiang Province, northeast of China.

Discussion section: In addition, we found that ICOS gene polymorphisms were associated with clinicopathologic features of breast cancer patients.

Conclusion section: This study first established that SNPs in the ICOS gene may affect breast cancer risk and some SNPs were also associated with clinical characteristics of breast cancer in Chinese women from northeast of China.

In Methods

1. Please provide the representative electrophoresis plots of PCR-RFLP.

Thank you for your suggestions, we combined gel pictures of five SNPs in one figure which we will submit in the Additional file1 as a supplemental figure. The submitted figure is as below (Figure S1):

Figure S1
Figure S1. Polymerase chain reaction–restriction fragment length polymorphism analysis of ICOS polymorphisms. (A) Rs11889031 SNP: lanes 1 and 4: homozygote CC, lane 2 and 3: heterozygote CT, lanes 5 and 6: homozygote TT. (B) Rs10932029 SNP: lanes 2, 5 and 6: homozygote TT, lane 1 and 4: heterozygote CT, lane 3: homozygote CC. (C) Rs4675374 SNP: lanes 1 and 6: homozygote TT, lane 2, 3 and 5: heterozygote CT, lane 4: homozygote CC. (D) Rs10183087 SNP: lane 1 and 2: homozygote AA, lane 3: heterozygote AC, lanes 4 and 5: homozygote CC. (E) Rs10932037 SNP: lane 1 and 3: homozygote CC, lane 2: heterozygote CT.


2. How did authors choose these five SNP? What is the rationale?

We selected SNPs according to the strategy below:

The SNPs we selected were potentially functional, as well as the most frequently studied in various diseases.
According to the reference, rs11889031 is situated in the NF-κB binding site and thereby affect affinities for NF-κB. Rs10932029 (IVS1+173) and rs4675374 are located in the intron1, the first intron contains various regulatory elements and splicing control elements. Mutations occurring in introns can induce the aberrant splicing due to the disruption of the splice site, the splicing enhancers and silencers, or the alteration of the pre-mRNA secondary structure, which results in translational prevention. Additionally, one study demonstrated that rs10932029 could influence the expression of CTLA-4 isoforms.

Rs10183087 (c.602A/C) and rs10932037 (c.1624C/T) are both located in the 3’-UTR of the ICOS. As known, untranslated regions of gene may influence the production of stable mRNA, the translational efficiency, the rate of mRNA decay and so on. Using the PupaSuite software, rs10183087 is speculated to be located in the exonic splicing enhancers (ESE), so it may influence mRNA splicing, eventually, affect protein function. Some previous studies also showed that rs10183087 was associated with delayed graft function. Rs10932037 was located in a MicroRNA-binding site, and one study had verified that rs10932037 can regulate the expression of ICOS mRNA. In addition, rs10932037 was proposed to be associated with kidney graft survival and the outcome of hematopoietic stem cell transplantation (HSCT).

Our research was to preliminarily investigate the association between potentially functional SNPs of ICOS gene and the risk of breast cancer. Therefore, we selected these five SNPs in our study.

3. No statistical power analysis was conducted. How did they determine the sample size?

Thank you for your questions.

Usually, to achieve convincing statistical support for a disease association, large sample size would be required. According to the minor allele frequencies of five SNPs (0.278 for rs11889031,
0.073 for rs10932029, 0.451 for rs4675374, 0.111 for rs10183087 and 0.035 for rs10932037) in NCBI, we calculated that a sample size of 141 cases and 152 controls (rs11889031), 354 cases and 387 controls (rs10932029), 131 cases and 139 controls (4675374), 251 cases and 273 controls (rs10183087), and 694 cases and 744 controls (rs10932037) could provide a power of 80% ($\alpha$ =0.05) to detect an OR of 2.0. There were 606 cases and 653 controls (11889031), 609 cases and 665 controls (rs10932029), 605 cases and 639 controls (4675374), 607 cases and 658 controls (rs10183087), and 608 cases and 651 controls (rs10932037) in our study population. Therefore, in addition to rs10932037, the sample size for other four SNPs had adequate statistical power. We selected rs10932037 as our research object according to following reasons:


However, considering the small sample size for rs10932037, false negative result may be exist, so further study using a large sample size is needed to confirm the association.
4. Before performing PCR-RFLP, they should have evaluated the accuracy of this method.

PCR-RFLP is likely to be false-negative or false-positive if unsuitable primes and restriction enzymes were selected. Were the PCR-RFLP results consistent with sequencing results?

How consistent?

Thank you for your advice.

The veracity of PCR-RFLP was more than 95% according to reporting of some references. In order to exclude the unsuitable primers, when we finished the PCR reaction, the sequences of some PCR products were confirmed by direct sequencing. Additionally, in order to confirm the accuracy of restriction enzymes, 10% samples of each SNP were randomly selected to be tested twice by different persons. Furthermore, 3% random samples of each SNP were confirmed by direct sequencing, and the reproducibility of both was 100%. Parts of the sequencing results are listed as below:

11889031 TT (Forward sequencing)

![Image 11889031 TT](attachment:image1)

11889031 CT (Forward sequencing)

![Image 11889031 CT](attachment:image2)

11889031 CC (Forward sequencing)

![Image 11889031 CC](attachment:image3)

Rs10932029 CT (Forward sequencing)

![Image Rs10932029 CT](attachment:image4)
Rs10932029 TT (Forward sequencing)

Rs10932029 CC (Forward sequencing)

Rs4675374 TT (Reverse sequencing)

Rs4675374 CC (Reverse sequencing)

Rs4675374 CT (Reverse sequencing)

Rs10183087 CC (Forward sequencing)

Rs10183087 AC (Forward sequencing)
5. What is the relationship between the five SNPs? Are they in LD?

Thank you for your questions.

Before doing the research, we used the information from HapMap Phase 2 data ([http://www.hapmap.org](http://www.hapmap.org)), and found that there was no LD between the five SNPs ($r^2 < 0.8$).

In Results

1. The haplotype analysis is somewhat weird. Does the combination of the five SNPs represent the haplotype of the ICOS gene? What the original mean of haplotype in genetics?

Thank you for your questions and advice.

A particular combination of alleles along a chromosome is termed a haplotype. Single SNP approaches were proved to be important for genetics-associated studies, but recent studies
suggested that analyses based on haplotypes would significantly improve the power of mapping
disease genes (Morris, R W, et al: On the advantage of haplotype analysis in the presence of

According to the references above, we further investigated the association of ICOS haplotypes with the risk of breast cancer. As a result, we found that two haplotypes of ICOS may play an important role in the development of breast cancer. Although the combination of five SNPs cannot represent the whole haplotype of the ICOS gene, it can reflect the combined effects of five SNPs on breast cancer risk.

How did they define the positivity of ER, PR, HER2, and p53?

Thank you for your questions.

In our study, the pathological and clinical information were obtained from medical files. The positivity or negativity of ER, PR, HER2, and P53 were from immunohistochemical results in medical files. The tumor was scored positive for ER and PR when >10% of tumor cells were stained (Zhang N, et al: BCL-2 (-938C > A) polymorphism is associated with breast cancer susceptibility. BMC Med Genet 2011,12:48). (Ding L, et al: Diverse Associations between ESR1 Polymorphism and Breast Cancer Development and Progression. Clin Cancer Res 2010, 16:
A case was considered positive when >10% of tumor cells were stained of nucleus (P53) or cell membrane (c-erbB-2) (Logullo AF, et al: **C-erbB-2 expression is a better predictor for survival than galectin-3 or p53 in early-stage breast cancer.** *Oncol Rep* 2007, **18**: 121-126).


3. The results were not adjusted for other risk factors (but only age) of breast cancer.

Thank you for your questions and advice.

We completely agree with this valuable suggestion by the reviewer. In the association studies on breast cancer, some risk factors such as age at menarche, menopausal status and environmental factors might act as potential confounders in the analysis. As a matter of fact, we attempted to carry out such an analysis before submitting the original manuscript, but when we collate these data, we found that some information was missing in some patients and controls, which limit the statistical power to analyze these factors together. As such we abandoned the attempt. It is a limitation in our study. Moreover, according to some references (Figueiredo JC, et al: **Polymorphisms cMyc-N11S and p27-V109G and breast cancer risk and prognosis.** *BMC Cancer* 2007, **7**: 99.), (Okobia MN, et al: **Leptin receptor Gln223Arg polymorphism and breast cancer risk in Nigerian women: A case control study.** *BMC Cancer* 2008, **8**:338.), the results were only adjusted for age, gender, the region and family history of breast or ovarian cancer. In our study, both of the cases and controls were Chinese Han women from Heilongjiang province of China. Additionally, the patients were self-reported no history of cancer, no metastasized cancer from other organs, and no previous radiotherapy or chemotherapy. The controls were also without any history of cancer. Therefore, the results were only adjusted for age.
4. No demographic information is presented for controls; no baseline comparison was performed between cases and controls.

Thank you for your suggestions.

In our research, the cases and controls were matched on the region, gender and age. Both of the cases and controls were Chinese Han women from Heilongjiang province of China, so we did not perform the comparison with gender or ethnicity between cases and controls. In addition, the age of cases and controls were matched. We used Chi-square test to analyze the age by SPSS software, the result was described in the table below.

<table>
<thead>
<tr>
<th>Age range (years old)</th>
<th>Study cohort</th>
<th>Validation cohort</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Case no (%)</td>
<td>Control no (%)</td>
</tr>
<tr>
<td>20~</td>
<td>32(5.25)</td>
<td>54(8.12)</td>
</tr>
<tr>
<td>45~</td>
<td>256(42.04)</td>
<td>259(38.95)</td>
</tr>
<tr>
<td>55~</td>
<td>111(18.23)</td>
<td>121(18.20)</td>
</tr>
<tr>
<td>65~</td>
<td>51(8.37)</td>
<td>42(6.32)</td>
</tr>
<tr>
<td>Total</td>
<td>609(100)</td>
<td>665(100)</td>
</tr>
</tbody>
</table>

We certified that the age of cases and controls in both study cohort and validation cohort was matched in our research according to the following reasons:

In the study cohort, the cases’ mean age was at 49.5 ± 10.1 years, and the controls’ mean age was at 48.0 ± 9.9 years, and the result of Chi-square test was that p-value = 0.151(p>0.05). In the validation cohort, the cases’ mean age was at 49.9± 10.2 years, and the controls’ mean age was at
48.9±10.0 years, and the result of Chi-square test was that p-value = 0.135 (p>0.05).

According to T-test, we found that p-value of mean age in both studies were more than 0.05.

Table 1

The positive rate of HER2 is high up to 30%, which is higher than previous data (about 20-25%).

Thank you for your questions.

Usually, over-expression of Her-2 has been shown to occur in ~20-30% of primary breast cancers (Slamon DJ, et al: Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. Science 1987, 235: 177-182). However, The significant difference was found in the expression of Her-2 between different populations (Choi DH et al: A comparison of five immunohistochemical biomarkers and HER-2/neu gene amplification by fluorescence in situ hybridization in white and Korean patients with early-onset breast carcinoma. Cancer 2003, 98(8):1587-1595). According to one book in Chinese (Xiyu Gong et al: breast pathology 2009, 423), the expression of HER2 protein can range from 17% to 35% in Chinese women. Additionally, one study evaluated breast cancer samples from 313 Chinese women, of which 100 (32%) were found to have Her-2 amplification (Beeghly-Fadiel A, et al: Her-2/neu amplification and breast cancer survival: results from the Shanghai breast cancer study. Oncol Rep 2008, 19(5):1347-1354). Another Study also revealed that the positive rate of Her-2 is 34.3% in Asian breast cancer patients (Selvarajan S et al: Over-expression of c-erbB-2 correlates with nuclear morphometry and prognosis in breast carcinoma in Asian women. Pathology 2006, 38(6):528-533). In our study, we have rechecked the expression of HER2 from medical files, the positive rate of Her-2 is 32.51%.
Table 2

No multiple-comparison correction is conducted. Are so-called “significant” results only because of false-positive?

Thank you for your kind advice and questions

We agree with the reviewer that the multiple-comparison correction is important in some studies. It can avoid type I error and decrease the false-positive rate. However, there is the possibility of over-correction, an increase of false-negative rate. Replication of study is another approach to find the true positive associations with the diseases (Chanock SJ, et al: Replicating genotype-phenotype associations. Nature, 2007, 447(7145):655-660).

The purpose of a replication study is to evaluate positive findings from a previous study, to provide credibility that the initial finding is valid. Replication is essential for establishing the credibility of a genotype–phenotype association, whether derived from candidate-gene or genome-wide association studies.

In our study, SNPs with suggestive statistical significance in the study cohort were replicated in an independent validation cohort to validate the results. We found that rs10932029 was associated with breast cancer risk and this association was also significant in the validation cohort. While further studies with confirmation of our results in other populations still be necessary.

Minor Essential Revisions

1. Why the “IFN-#”, “ICOS”, “CTLA-4” in sentence “population produced higher IFN-#, indicating that ICOS interacts with CTLA-4 and plays an important role in tumor immunity” were all in italic. They are protein rather than gene in the context.

Thank you for your helpful suggestions.
We are so sorry to make such clerical errors, and we have revised them in the revised manuscript as below (words in blue).

Treated with CTLA-4 blockade, ICOS expressed higher on CD4$^+$ T cells from peripheral blood and tumor tissues of bladder cancer patients and this CD4$^+$ICOS$^{hi}$ T cell population produced higher IFN-γ, indicating that ICOS interacts with CTLA-4 and plays an important role in tumor immunity.

3. In Supplemental Table 1, the results are incomplete.

Thank you for your kind suggestions.

In the supplemental table 1, we only show the positive results. For the association between rs10932029 (IVS1+173) and the status of C-erbB-2, we did not provide the association with alleles. In order to describe the results more clearly, we add the analysis of the association between alleles in rs10932029 and C-erbB-2 to the Additional file 2, Table S1. The revised table is as below (words in blue):

Table S1. ICOS polymorphisms and clinical features in cases in the study cohort and validation cohort

<table>
<thead>
<tr>
<th>Clinical features</th>
<th>SNP and allele</th>
<th>Genotype</th>
<th>Study cohort</th>
<th>Validation cohort</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Positive (%)</td>
<td>Negative (%)</td>
</tr>
<tr>
<td>C-erbB-2</td>
<td>rs10932029</td>
<td>TT</td>
<td>178(89.45)</td>
<td>266(83.91)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CT</td>
<td>19(9.55)</td>
<td>50(15.77)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CC</td>
<td>2(1.01)</td>
<td>1(0.32)</td>
</tr>
<tr>
<td>T</td>
<td></td>
<td></td>
<td>375(94.22)</td>
<td>582(91.80)</td>
</tr>
<tr>
<td>C</td>
<td></td>
<td></td>
<td>23(5.78)</td>
<td>52(8.20)</td>
</tr>
</tbody>
</table>

In Supplemental Table 1, the results are incomplete.

Thank you for your kind suggestions.

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<td>1(0.32)</td>
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<td>T</td>
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<td></td>
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<td>582(91.80)</td>
</tr>
<tr>
<td>C</td>
<td></td>
<td></td>
<td>23(5.78)</td>
<td>52(8.20)</td>
</tr>
<tr>
<td>PR</td>
<td>rs11889031</td>
<td>CC</td>
<td>171(44.88)</td>
<td>38(29.23)</td>
</tr>
<tr>
<td>------</td>
<td>------------</td>
<td>-------</td>
<td>------------</td>
<td>-----------</td>
</tr>
<tr>
<td>CT</td>
<td></td>
<td></td>
<td>168(44.09)</td>
<td>76(58.46)</td>
</tr>
<tr>
<td>TT</td>
<td></td>
<td></td>
<td>42(11.02)</td>
<td>16(12.31)</td>
</tr>
<tr>
<td>C</td>
<td></td>
<td></td>
<td>510(66.93)</td>
<td>154(58.78)</td>
</tr>
<tr>
<td>T</td>
<td></td>
<td></td>
<td>252(33.07)</td>
<td>108(41.22)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Lymph node involvement</th>
<th>PR=progesterone receptor; OR= odds ratio; CI = confidence interval</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>The P-values was calculated using the Chi-square test.</td>
</tr>
</tbody>
</table>

**Level of interest:** An article whose findings are important to those with closely related research interests

**Quality of written English:** Needs some language corrections before being published

Thank you for your suggestions.

We have corrected some language in the revised manuscript.

**Statistical review:** No, the manuscript does not need to be seen by a statistician.
Declaration of competing interests:

I declare that I have no competing interests

Thank you very much for your continued attention.

Best wishes,

Sincerely yours,

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Fax: +86-451-86697322

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