Peer Review File

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Reviewer Comments

The paper titled "KRAS, YWHAE, SP1 and MSRA as biomarkers in endometrial cancer" is interesting, which conduct a prospective study to delineate the role of the DEPs like KRAS, MSRA, SP1 and YWHAE as focused biomarkers, and even regarding KRAS in predicting standard individual treatment approach of cancer progression after hyperplasia with or without atypia in endometrium. However, there are several minor issues that if addressed would significantly improve the manuscript.

Comment 1:

(1) How to assess the specific molecular characteristics of endometrial cancer?

Reply 1(1):

Changes in the text: We have modified our text as advised: see Page 4 Line 14-16, Page 5 Line 1-4:

The Cancer Genome Atlas (TCGA) classifies EC into four groups, each of which is based on different histopathology or molecular sub-type, as well as prognostic potential: Group 1, polymerase epsilon (POLE), ultramutated, associated with good prognosis; Group 2, microsatellite instability (MSI) hypermutated; Group 3, copy-number low (CN-Low) endometrioid, group 2 and 3 shown similar progression-free survival rates; Group 4, copy-number high (CN-High), serous-like, with worse prognosis.

(2) What is its potential clinical utility in early detection, disease risk stratification, and guidance of targeted therapy?

Reply 1(2):

Potential clinical utility in early detection, disease risk stratification:

We have mentioned in our text: see Page 32 Line 8-11:

KRAS plays the important role of predicting early checkpoint of transition from hyperplastic endometrium to early-stage well-differentiated (grade I) oestrogen-related EC, as well as further transition from low-grade to high- grade type I EC.

See Page 36 Line 2-6:

As to uterus tumor, YWHAE-NUTM2A/B endometrial stromal sarcomas (ESS) is a recently described variant of high-grade endometrial stromal sarcomas (HG-ESS) which is included in the 2014 WHO Classification of Tumors of the Female Reproductive Organs excluded from the prior WHO 2003 Classification.

Guidance of targeted therapy:

Changes in the text: We have modified our text as advised: see Page 5 Line 4-14:

Also new biologic and molecular therapies for the treatment of endometrial carcinoma are being assessed in clinical trials. Application of TCGA classification may help in deciding the use of immunotherapy with immune checkpoint inhibitors like

anti-programmed cell death-1(PD-1)/programmed cell death-Ligand 1(PD-L1) treatment including Lenvatinib/pembrolizumab for TMB-H [≥10 mutations/megabase (mut/Mb)] or MSI-high/mismatch repair (MMR) deficient tumors (The multicohort phase Ib KEYNOTE-028 study).NCCN also recommended biomarker-directed systemic therapy for second-line treatment for EC like bevacizumab (randomized phase II trial Gynecologic Oncology Group trial), Nivolumab, Larotrectinib or entrectinib for Neuro Trophin Receptor Kinase (NTRK) gene fusion-positive tumors.

Comment 2:

(1) In addition to the liquid chromatography-tandem mass spectrometry technology used in this study, are there other technologies used in the screening of biomarkers for endometrial cancer?

Reply 2(1):

We have mentioned in our text: see Page 7 Line 4-7:

Yes, RNA sequencing analysis and Affymetrix Single nucleotide polymorphisms (SNP) microarrays can be used in the screening of biomarkers for endometrial cancer.

(2) If so, what are the differences and advantages?

Reply 2(2):

Changes in the text: We have modified our text as advised: see Page 7 Line 4-10:

Unlike studies that focus on RNA sequencing analysis, which can only detect gene expression alterations, or Affymetrix Single nucleotide polymorphisms (SNP) microarrays used in biospecimens to analyse mRNA, miRNA and methylation data which cost high, TMTs enables relative peptide and protein quantification across analyzed samples as means of identifying differential expression and can therefore provide insight into the proteolytic activities occurring within a complex biological sample.

Comment 3:

The diagnosis of endometrial cancer relies on the observation of tumor cells in endometrial aspiration biopsy. How to reduce the missed diagnosis rate and histological error typing and grading?

Reply 3:

We also conducted IHC to exam biomarkers in endometrial cancer, and endometrial specimens were randomly examined by two independent investigators to reduce the missed diagnosis rate and histological error typing and grading.

We have mentioned in our text: see Page 18 Line 4-10, Page 19 Line 1-2:

Immunohistochemical staining (IHC) was conducted to exam kirsten rat sarcoma viral oncogenes homologue (KRAS), methionine sulfoxide reductase A (MSRA), tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein epsilon (YWHAE) and trans-acting transcription factor 1 (SP1) expression in EC (n=17) and atypical hyperplasia (n=3) sections from hysterectomy, as well as secretory phase endometrium specimens (n=2) from curettage in Shanghai General Hospital affiliated

to Shanghai Jiao Tong University. Xenograft tumor tissue was excised from mice sacrificed by cervical dislocation, endometrial specimens were randomly examined by two independent investigators.

Comment 4:

The research progress of endometrial cancer-related tumor markers and how to provide effective help for early clinical diagnosis, targeted therapy and improvement of patient prognosis should be included in the discussion.

Reply 4:

Changes in the text: We have modified our text as advised: see Page 32 Line 12-14:

The research progress of endometrial cancer-related tumor biomarkers may carry important advantages in clinical practice of possible targeted therapy to endometrial cancer patients in order to improve patient prognosis.

We have mentioned in our text:

see Page 32 Line 14-16, Page 33 Line 1-5:

A series of studies were devoted to inhibit KRAS mutations including KRAS direct binding molecules, KRAS membrane localization targeting enzymes, or downstream signaling, synthetic lethal interactors, inhibiting KRAS gene expression, though immune system pathways. Recent studies have also shown that a combination therapy of mitogen-activated extracellular kinase (MEK) inhibitors plus anti-oestrogen agents may alter oestrogen signaling in KRAS-mutant EC and thus improve the response rate.

See Page 37 Line 7-10:

KRAS mutations can cause resistance to epidermal growth factor receptor (EGFR) inhibitors. Thus KRAS mutation has emerged as the major negative predictive biomarker for response to anti-EGFR chemotherapy agents in colorectal cancer patients.

See Page 36 Line 2-6:

As to uterus tumor, YWHAE-NUTM2A/B endometrial stromal sarcomas (ESS) is a recently described variant of high-grade endometrial stromal sarcomas (HG-ESS) which is included in the 2014 WHO Classification of Tumors of the Female Reproductive Organs excluded from the prior WHO 2003 Classification.

See Page 37 Line 10-16:

YWHAE-NUTM2A/B fusion subsequent to a t(10;17) (q22;p13) has been associated with a more aggressive neoplasm and a poorer prognosis when compared to its low-grade counterpart in HG-ESSs. YWHAE translocation correlated with low mitotic index and improved prognosis of undifferentiated uterine sarcomas. YWHAE (14-3-3ε) expression is predictor of clinical outcome in a large dataset of myeloma patients receiving Bortezomib (BTZ) as first line therapy.

Comment 5:

How to integrate molecular characterization of endometrial cancer into clinicopathological analysis to develop predictive biomarkers?
Reply 5:
Changes in the text: We have modified our text as advised: see Page 38 Line 10-12:
Clinicopathological immunohistochemical staining could be conducted to exam these biomarkers in patients' endometrial tissue to develop predictive outcome.

Comment 6:
Please give the full names of these abbreviations (SDS-PAGE, DTT, and so on) when they first appeared. Please check carefully. There are many similar cases in the paper.
Reply 6:
Changes in the text: We have modified our text as advised: see Page 4 Line 10-11:
International Federation of Gynecology and Obstetrics (FIGO)

see Page 4 Line 14-16, Page 5 Line 1-10:
The Cancer Genome Atlas (TCGA)
polymerase epsilon (POLE)
microsatellite instability (MSI)
Copy-number low (CN-Low)
Copy-number high (CN-High)
anti-programmed cell death-1(PD-1)/programmed cell death-Ligand 1(PD-L1)
TMB-H: mutations/megabase (mut/Mb)
MMR: mismatch repair
NeuroTrophin Receptor Kinase (NTRK)

see Page 7 Line 6:
Affymetrix Single nucleotide polymorphisms (SNP) microarrays

see Page 11 Line 5, 12, 14:
SDT buffer [4% Sodium dodecyl sulfate (SDS), 100 mM Tris–HCl]
bicinchoninic acid (BCA) Protein Assay kit
SDS-polyacrylamide gel electrophoresis (PAGE)

see Page 7 Line 6,10, Page 13 Line 5
dithiothreitol (DTT)
indole-3-acetic acid (IAA)
tetraethylammonium bicarbonate (TEAB)

see Page 14 Line 13-14:
reversed-phase liquid chromatography (RPLC)

see Page 18 Line 4-7:

kirsten rat sarcoma viral oncogenes homologue (KRAS)

methionine sulfoxide reductase A (MSRA)

tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein epsilon (YWHAE)

trans-acting transcription factor 1 (SP1)

see Page 16 Line 20:
Paired box-8 (PAX-8), Mutl homolog l gene (MLH1)

see Page 34 Line 1:
glypican (GpC)-rich promoter regions

see Page 37 Line 16:
Bortezomib (BTZ)

Comment 7:
The function and mechanism of KRAS, YWHAE, SP1 and MSRA should be added, which may be more meaningful.
Reply 7:
Changes in the text: We have mentioned in our text:
KRAS:
see Page 31 Line 7-11:

KRAS mutations promote the down-regulation of membrane receptor signaling through mitogen-activated protein kinase (MAPK) and phosphoinositide-3-kinase/v-akt murine thymoma viral oncogene (PI3K/AKT) pathways, which result in dependent autophagy that is necessary for cancer progression as well as promote proliferation and subsequent carcinogenesis.

see Page 31 Line 23:

KRAS mutants knock-out leads to inhibition of upstream signaling pathways.

see Page 32 Line 8-11:

KRAS plays the important role of predicting early checkpoint of transition from hyperplastic endometrium to early-stage well-differentiated (grade I) oestrogen-related EC, as well as further transition from low-grade to high- grade type I EC.

SP1:
see Page 34 Line 3-9:

Dysregulation of SP1 has been found to be involved in many cancers, and results in the suppression of cell migration and invasion in squamous cervical cancer, promotes the migration of ovarian cancer cells, as well as stimulated stem cell of colon cancer growth and induce apoptosis. Additionally, SP1 knockdown could reverse the effects of miR-490 inhibition on the malignant behaviors of ishikawa cells

and inhibited PI3K/AKT pathway which elucidated the roles SP1 axis in EC development and may provide a new strategy for EC therapy.

MSRA:
see Page 34 Line 1, Page 35 Line 1-3:

MSRA is located on chromosome 8p23.1 and encodes the methionine sulfoxide reductase, which is known to protect proteins from oxidation and acts as a reactive oxygen species (ROS) scavenger. MSRA performed a tumor-suppressive effect in both lung squamous carcinoma and adenocarcinoma than in adjacent normal tissues.

see Page 37 Line 3-5:

KRAS, SP1 and YWHAE are all associated with PI3K/ AKT/mTOR pathway which are found implicated in EC pathogenesis.

YWHAE:
see Page 20 Line 6-13:

YWHAE functions as a molecular framework to coordinate cellular signaling by binding to phosphoserine- or phosphothreonine-containing proteins. As to uterus tumor, YWHAE-NUTM2A/B endometrial stromal sarcomas (ESS) is a recently described variant of high-grade endometrial stromal sarcomas (HG-ESS) which is included in the 2014 WHO Classification of Tumors of the Female Reproductive Organs excluded from the prior WHO 2003 Classification. YWHAE interactome in myeloma cells also revealed enrichment in PI3K-AKT-mTOR.

see Page 36 Line 12-16, P37 Line 1-3:

YWHAE lncRNA downregulation or upregulation induced corresponding a significant downregulation or upregulation of K-Ras gene at the mRNA level, YWHAE encoded lncRNA promotes activation of K-Ras/Erk1/2 and PI3K/Akt signaling pathways in HCT116 cells. Starbase data also showed positive correlation between YWHAE gene and K-Ras gene in colorectal cancer tissues. Specifically, positive effect of MsrA-dependent interaction on the ubiquitination of 14-3-3ζ through MSRA knockout mice exhibiting high levels of 14-3-3ζ compared with the corresponding wild-type strain.

Comment 8:
The current status of biomarker-driven targeted therapy in endometrial cancer and the basic principles of ongoing clinical trials with new targeted drugs should be included in the introduction.
Reply 8:
Changes in the text: We have modified our text as advised: see Page 5 Line 4-14:

Also new biologic and molecular therapies for the treatment of endometrial carcinoma are being assessed in clinical trials. Application of TCGA classification

may help in deciding the use of immunotherapy with immune checkpoint inhibitors like anti-programmed cell death-1(PD-1)/programmed cell death-Ligand 1(PD-L1) treatment like Lenvatinib/pembrolizumab for TMB-H [≥10 mutations/megabase (mut/Mb)] or MSI-high/mismatch repair (MMR) deficient tumors (The multicohort phase Ib KEYNOTE-028 study). NCCN also recommended biomarker-directed systemic therapy for second-line treatment for EC like bevacizumab (randomized phase II trial Gynecologic Oncology Group trial). Nivolumab, Larotrectinib or entrectinib for NeuroTrophin Receptor Kinase (NTRK) gene fusion-positive tumors.