Author's response to reviews

Title: Long term survival following the detection of circulating tumour cells in head and neck squamous cell carcinoma

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Author's response to reviews:

Dear Dr Norton,

Thank you for reviewing our manuscript entitled “Long term survival following the detection of circulating tumour cells in head and neck squamous cell carcinoma” by Winter, Stephenson, Subramaniam, Paleri, Ha, Marnane, Krishnan and Rees.

We would like to thank all 5 reviewers for their constructive comments and suggestions and have addressed these in a new draft of our manuscript that we would now like to resubmit for your consideration for publishing in BMC Cancer. Please find following a summary of the changes we have made as per each reviewer.

We thank you for your consideration of this manuscript and look forward to the outcome of your review process.

Yours Sincerely,

Sally Stephenson

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RESPONSES TO REVIEWERS

Reviewer – Thomas Brunner

1. Query re: Abstract

Samples were collected from all patients both before their surgery and after. To clarify this, the abstract has been reworded. “Seven patients were positive for circulating tumour cells both prior to and after surgery, 4 patients were positive prior to but not after surgery, 3 patients were positive after but not prior to surgery and 2 patients were negative. Two patients tested positive for circulating cells but there was no other evidence of tumor spread.”

2. Query re: Conclusion

Our results show that the immunomagnetic enrichment procedure can identify circulating cells from patients with advanced head and neck tumours. This clearly demonstrates the clinical utility of immunobead RT-PCR as a minimally invasive technique that facilitates the rapid screening of patient peripheral blood for CTCs associated with head and neck cancers, and therefore the potential for developing this assay for future incorporation into the clinical setting.

3. Query re: Materials and Methods - Reconstruction experiments using 10, 50, 100 and 500 cells per 10 ml.

We have included a recent reference that determined the number of circulating tumour cells in head and neck patients. “A recent study by Balasubramanian et al (2009) determined that the number of circulating tumour cells in head and neck patients ranged from 0 to 214 per ml therefore the range we have chosen for these reconstruction experiments will be suitable to determine the lower limit of detection of HNSCC.” Balasubramanian et al (2009) Confocal images of circulating tumor cells obtained using a methodology and technology that removes normal cells. Mol Pharm. May 15. [Epub ahead of print].

4. Table 1 – adjuvant therapy was heterogeneous

We agree that the patient group is heterogeneous regarding tumour site, stage and therapy. At the time of entry into the study, we did not know the end histological tumour stage, nor the eventual treatment plan. The clinical management of these patients is adjusted according to the results of surgery and the wishes of the patient. The main aim of the study was to assess the ability of the immunomagnetic bead technique to detect circulating tumour cells in head and neck cancer, and then to see if there was any association between any of the cell surface markers and outcome. This is not aimed at being a definitive study to show the benefit of any one marker, but to allow future studies to expand
further our understanding of the clinical significance of circulating tumour cells in Head and Neck cancer.

5. Table 3

BerEP4 is the antibody that is conjugated to the immunobeads and this antibody recognizes the EpCam protein on the surface of epithelial cells. This has been clarified in the text on page 4 - “The BerEP4 antibody recognises the EpCam protein expressed on the surface of epithelial cells.” The synonyms for EpCam and EMA have also been provided in the Table 3 text as requested – “The BerEP4 antibody recognises the EpCam protein and the EMA antibody recognises Muc1/EMA/Mucin 1.”

We used beads labeled with antibodies specific to two epithelial proteins to pull out epithelial cells which were then identified using markers to four genes that are commonly over-expressed in head and neck tumour cells. Commercially labeled beads are fully optimized for such experiments and labeling is subjected to rigorous quality control for this purpose. In future, beads labeled with antibodies to other surface proteins, including perhaps EGFR and EphB4 may find application but have not yet been tested.

6. Figure 1, Table 4.

The details of figure 1 are summarized in Table 3. The typographical error on Page 11, line 8 referring to Table 4 has been corrected. The figure caption has been altered to include how the cells with beads attached were counted – “The number of cells with beads attached and the number of beads attached per cell were counted by eye at 10X magnification”. We did not use a negative control cell line as beads labeled with both of these antibodies have been fully tested in other studies. We did not do a FACS analysis because the purpose of this experiment was simply to confirm that BerEP4-labelled beads were the most appropriate to use in the isolation of circulating head and neck cells and we planned to use commercially labeled beads for the patient sample tests.

7. Page 12 – Was the blood drawn from an artery or vein?

For the reconstruction experiments venous peripheral blood was used. For the patient samples, arterial peripheral blood was used. To clarify this we have included “Peripheral blood was collected into dipotassium EDTA tubes from an arterial line at the commencement and end of surgery. For the reconstruction experiments, venous peripheral blood samples were collected from normal individuals, also into dipotassium EDTA tubes” in the Materials and Methods section.

8. Page 13 – Did we check for the influence on the overall survival in the six patients who were positive for three markers?

When considering the 6 patients (Patients 6, 7, 8, 11, 13 and 15) who were positive for the three markers, (ESX, EGFR and EphB4) there was no significant correlation with overall survival, p=0.22). This has been included in the results for
9. Page 13 – Calculate the statistical degree of agreement between the expression of markers in duplicate samples.

The agreement between the expression of markers in the duplicate samples was tested using a 2-tailed paired T-test.

This has been reported in the results section on page 13 as “Duplicate samples were analysed for agreement using a 2 tailed paired t-test. There was a significant correlation between the samples taken pre-operatively for the antigen ESX only, (p=0.02).”

10. Section on page 14 comparing individual marker expression prior to and after surgery

The relevance of this section is that patients who are negative prior to surgery might be positive post-surgery simply due to the manipulation of the tissue. We will leave this section in but have shortened the paragraph and referred instead to Table 5.

Reviewer – Jeffrey Chalmers

1. With regard to comments about the heterogeneity of gene expression within a cell line population, we will include the reviewer’s own reference Tong et al (2007) as he has suggested.

2. Quantification of contaminating PBLs.

In a previous publication, a member of this team has already reported experiments validating the use of this technique and these markers for the specific detection of a single cancer cell, even if co-isolated with 100 mononuclear cells Raynor et al (2002) Biomed Central – Cancer 2:14. As reported by de Cremoux et al (2000) Clin Cancer Res 2000, 6:3117-3122, this is at least 10-fold the number of cells that might actually contaminate the bead pellet and for this reason we did not consider it important to quantitate mononuclear cells in this assay. The reference to de Cremoux et al (2000) has been added.

2. The aim of this study was to determine whether samples from patients with head and neck cancers were positive or negative for circulating cells, not quantify the number of cells that were found. Our “spiking” experiment was performed to ensure that our assay would be sufficient to identify circulating cells if they are present in the sample. Given that it has been estimated that patients with head and neck cancers typically have 1-10 tumour cells / 106 mononuclear cells, and that there are 106 mononuclear cells in 0.1 – 0.4 ml of whole blood, we would expect that by examining 10 ml samples of patient blood, we might expect positive samples to contain in the order of 25 – 1000 cells. This is well within the sensitivity of our method.
3. Reviewer’s Johannes Huelsenbeck and Thomas Brunner have also suggested that the results section describing detection of circulating cells prior to and after surgery was long and hard to read and we have accordingly shortened this paragraph (see answer to specific comment by this reviewer) but would prefer to leave the rest of the results section as written.

4. The term “reconstruction experiments” has been used previously in several articles.

However we have added the word “spiking” to the headings in the Materials and Methods section and the Results section that refer to these experiments.

5. Regarding the protocol used to process the patient samples.

The CELLection beads were used to process the patient samples as per the statement in the Materials and Methods section – “The immunobead RT-PCR technique was performed as described above using the beads commercially labelled with the BerEP4 antibody, CELLection Epithelial Enrich Dynabeads (Dynal, Invitrogen), to ensure optimal isolation of circulating HNSCC expressing the EpCam protein.” This was the same method that was used for the reconstruction experiments.

Reviewer – Daniel Zips

1. The aim of the study has been clarified in the introduction.

“The aim of this study was to develop an assay for the detection of circulating tumor cells (CTCs) originating from head and neck squamous cell carcinomas and test the utility of this assay this in a pilot study using peripheral blood samples from individuals with advanced disease.”

2. Re: small sample size, large heterogeneity in clinical and treatment parameters and insufficient consideration of established predictors of distant metastasis.

This issue has been addressed previously however to further clarify, these patients were a cohort drawn from the routine care of head and neck cancer patients presenting to the Royal Adelaide Hospital, South Australia, Australia. As such, their management is decided upon by a Multidisciplinary Clinic prior to commencement of therapy. This is then adjusted by the Clinic as details of histologic results of surgery become known. In addition, patients wishes are taken into account, which will influence therapy, and this will change throughout the period of care. The patient may also be affected by morbidity related to treatment which may affect ability to undergo the whole course of radiotherapy, or chemotherapy. These factors are recognised in the difficulty delivering optimal treatment for head and neck cancer patients and reflects the actuality of treatment. It is recognised that in many published studies, these factors do not appear as these patients will be precluded from the study, but represent the reality of life in care delivery.
The philosophy of the RAH MDC is that patients with resectable disease are offered surgery and that patients with advanced disease (stage III/IV) are then offered adjuvant post operative radiotherapy. There has been an increasing tendency to offer post operative concomitant chemo-radiotherapy in light of the NEJM articles published on this level of care, for specified patients. The use of EGFR related therapy was not a part of our planned treatment for any patient during the course of the study. At the time of entry into the study, we did not know the end histological tumour stage, nor the eventual treatment plan or the ability or desires of the patient to undergo such a plan.

As stated previously, we agree that the patient group is heterogenous regarding tumour site, stage and therapy but as the main aim of the study was to assess the ability of the immunomagnetic bead technique to detect circulating tumour cells in head and neck cancer, and then to see if there was any association between any of the cell surface markers and outcome. This is not aimed at being a definitive study to show the benefit of any one marker, but to allow future studies to expand further our understanding of the clinical significance of circulating tumour cells in Head and Neck cancer.

3. Loco-regional control by optimized radiotherapy ....

Page 3, Paragraph 1. The wording ‘Advances in surgery and radiotherapy over the past few decades have resulted in improved loco regional control however this has not translated to an improved overall survival.’ Has been changed to ‘Advances in surgery and radiotherapy over the past few decades have resulted in improved loco regional control however this has not translated to an improved overall survival in all studies.’

Reviewer - Johannes Huelsenbeck

1. Amplification of marker genes in reconstruction experiments was tested using 40 PCR cycles in a Perkin Elmer PTC100. Samples were analysed by gel electrophoresis. The aim of this experiment was to prove that marker gene expression could be detected after immunobead isolation of circulating tumour cells. The patient samples were analysed in a Corbett Rotorgene 3000 and 50 cycles were chosen so that all positive samples would reach plateau. Peripheral blood samples from normal individuals and normal samples spiked with known numbers of tumour cell lines were used as negative and positive controls respectively. This has been clarified in the Materials and Methods section.

2. The comment regarding expression of ELF3/ESX in head and neck cancer cell lines has been altered to “ELF3/ESX expression was also detectable in both cell lines”.

3. The results section describing detection of circulating cells prior to and after surgery has been shortened as requested. “Not all patients who were positive before surgery were also positive after surgery and vice versa. Furthermore often only one of the two samples collected either prior to or after surgery was positive.
The results are summarised in Table 5."

4. Figure 2 has been improved as requested.

5. The data presented in Figure 5 was generated using a real-time PCR machine and products were not analysed by gel electrophoresis.

6. Table 3 – we have included the first digit after the decimal point and standard deviations for all values (n = 4).

Reviewer – Frank Pajonk

1. “Manuscript is written sloppy (sic)”

We have already altered the results section as suggested by other reviewer’s but do not feel the manuscript needs further re-writing. All references have been cross-checked.

2. Re: Peripheral blood samples from normal individuals

Normal blood samples from blood donors were provided by the Australian Red Cross Blood Service, Adelaide, South Australia. Information about these individuals was not available to us but all samples are collected for use in blood transfusions etc. and are therefore considered as normal as possible.

3. Re: cell lines used in each experiment.

Specific cell lines used in optimization assays are already specified in the Materials and Methods and Results sections. In some experiments representative cell lines were used and this is already specified in the appropriate places in the text.

4. Re: Suggestion that “CTC are most likely contaminating skin cells”.

We are confident that cells identified in this Immunobead RT-PCR assay are NOT contaminating skin cells for two reasons. Firstly, when the peripheral blood samples are collected the first few ml are discarded and with this would be any contaminating skin cells. Secondly, it should be noted that keratinocytes are not EpCam positive (American Journal of Pathology. 2003;163:2139-2148) and therefore would not be isolated using BerEP4-conjugated immunobeads.

5. Statistics – “continuous variables”, Cox model, Log rank test

Within the statistical model the following variables were considered in the Cox proportional hazard model – Age, Sex, T stage, N stage and antigen expression. This has been included in the method section describing the statistics.