A distinct pre-existing inflammatory tumour microenvironment is associated with chemotherapy resistance in high-grade serous epithelial ovarian cancer

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Background: Chemotherapy resistance is a major determinant of poor overall survival rates in high-grade serous ovarian cancer (HGSC). We have previously shown that gene expression alterations affecting the NF-κB pathway characterise chemotherapy resistance in HGSC, suggesting that the regulation of an immune response may be associated with this phenotype.

Methods: Given that intrinsic drug resistance pre-exists and is governed by both tumour and host factors, the current study was performed to examine the cross-talk between tumour inflammatory microenvironment and cancer cells, and their roles in mediating differential chemotherapy response in HGSC patients. Expression profiling of a panel of 184 inflammation-related genes was performed in 15 chemoresistant and 19 chemosensitive HGSC tumours using the NanoString nCounter platform.

Results: A total of 11 significantly differentially expressed genes were found to distinguish the two groups. As STAT1 was the most significantly differentially expressed gene (P = 0.003), we validated the expression of STAT1 protein by immunohistochemistry using an independent cohort of 183 (52 resistant and 131 sensitive) HGSC cases on a primary tumour tissue microarray. Relative expression levels were subjected to Kaplan–Meier survival analysis and Cox proportional hazard regression models.

Conclusions: This study confirms that higher STAT1 expression is significantly associated with increased progression-free survival and that this protein together with other mediators of tumour–host microenvironment can be applied as a novel response predictive biomarker in HGSC. Furthermore, an overall underactive immune microenvironment suggests that the pre-existing state of the tumour immune microenvironment could determine response to chemotherapy in HGSC.

Epithelial ovarian cancer is a leading cause of morbidity and mortality from gynaecologic malignancies, with ~22,000 new cases each year in North America. High-grade serous ovarian cancer (HGSC) is the most prevalent histological type characterised by late detection, metastasis and resistance to chemotherapy (Marcus et al, 2014). Approximately 50% of patients exhibit resistance to chemotherapy within 6 months of treatment that eventually results in death because of tumour relapse.

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A. A number of reports (Sakamoto et al, 2001; Selvanyagam et al, 2004; Bernardini et al, 2005), aimed at identifying mechanisms leading to primary chemoresistance, have revealed differential gene expression signatures in the drug-resistant patients. Recently, we showed that gene expression alterations in chemotherapy-resistant HGSC affected, among others, the NF-κB pathway, drawing attention for a need to understand immunoregulation in the context of differential drug response in this tumour (Koti et al, 2013).

Chemotherapy resistance can be defined as being either primary (innate) or secondary (acquired). Primary chemotherapy resistance is conferred by pre-existing gene expression that is inherent within the tumour, in chemotherapy-naïve tumour cells or in the immediate microenvironment, that provides a state of resistance to a range of drugs. Secondary chemotherapy resistance is conferred to the tumour as a direct result of alterations that are acquired or induced by selection following exposure to chemotherapeutic agents. Chemotherapy resistance remains a major determinant of poor survival rates in HGSC, but there has been little progress in developing novel biomarkers of therapeutic response. This failure may partially be attributed to most studies being directed towards the tumour cell-intrinsic events and ignoring the contributory effects of variation in the local immune response. The role of the tumour microenvironment in the survival of tumour cells, and the dual roles of cancer immunediting via tumour-promoting inflammation and suppression, is becoming well recognised (Zhang et al, 2003; Galon et al, 2006; Schreiber et al, 2011; Baxevanis et al, 2013). There is also increasing awareness that pre-existing adaptive immune status affect response to subsequent therapy across various cancers. A diverse tumour immunome was recently shown to be associated with varying clinical outcomes in colorectal cancer (Bindea et al, 2013). The abundance of tumour-infiltrating lymphocytes (TILs) correlates with survival across multiple cancers. In HGSC, ratios of CD8+ T cells to T regulatory cells have been associated with disease outcome (Preston et al, 2013). Higher survival rates have been reported in tumours containing both CD8+ T and CD20+ B cells (Milne et al, 2009; Nelson, 2010). In addition to the cellular infiltration, recent studies also suggest a significant role of CXC chemokines that recruit TILs in ovarian cancer (Kryczek et al, 2009; Rainczuk et al, 2012). Although a number of studies have addressed the immune cell infiltration in the HGSC tumour microenvironment, there is insufficient information on the role of cytokine/chemokine mediators that putatively lead to a variable adaptive immune response to HGSC.

Based on our previous findings implicating NF-κB to be one of the key pathways involved in chemotherapy resistance, we hypothesised that a pre-existing differential tumour immune microenvironment might lead to a variable response to chemotherapy in HGSC. We therefore analyzed differences in the tumour inflammatory environment using digital multiplexed gene expression profiling in a cohort of sensitive and resistant HGSC tumours, with an overall objective of identifying predictive markers of response to primary chemotherapy resistance. These data suggested involvement of T helper type 1 (Th1)-related factors such as signal transducer and activator of transcription 1 (STAT1), and other factors associated with an adaptive immune response that could be associated with the drug response phenotype as this protein is a key mediator of Th1 response following interferon stimulation. The rationale for determining STAT1 expression in tumour tissues was because variation in cytokine activation and expression could arise from both tumour and a variety of the microenvironment cellular populations. Solid tumours contain a diversity of cell types recruited into close proximity (reviewed in Junittila and de Sauvage, 2013), so that overall immunohistochemical quantification of STAT1 was performed. The immunohistochemistry results were obtained from an independent cohort of chemo-naïve HGSC and they confirmed that STAT1 protein levels were significantly associated with progression-free survival, and implicate a role of the inflammatory tumour microenvironment in mediating chemotherapy response in this tumour.

**MATERIALS AND METHODS**

**Ethics statement.** This study was approved by Queen’s University Institutional Ethics Review Board, the CHUM institutional ethics committee (Comité d’éthique de la recherche du Centre hospitalier de l’Université de Montréal) and Ottawa Health Research Institute (OHRI) Research Ethics Board. Informed consent from all patients was obtained before sample collection.

**Patient samples for discovery study.** Fresh frozen tumour tissue samples from a cohort of 34 locally advanced (stage IIA–IV) HGSC tumours were accrued from the Ontario Tumour Bank (Ontario Institute for Cancer Research) and the OHRI. All samples were collected from patients who were naïve to chemotherapy and radiotherapy at the time of primary cytoreductive surgery. Samples were stored at −80°C immediately following collection until further processing. All samples had ≥70% tumour content and were histologically classified using WHO criteria, and disease staging was performed as per the International Federation of Gynaecology and Obstetrics (FIGO) guidelines. Our discovery cohort consisted of 15 patients who exhibited progressive disease within 6 months of initiation or within 8 months of completion of chemotherapy classified as intrinsically resistant/partially resistant (R/PR) and 19 patients showing no relapse until 18 months considered intrinsically sensitive (S) to chemotherapy (Koti et al, 2013; Park et al, 2013).

**NanoString-based gene expression profiling.** Total RNA was isolated from fresh frozen tissue samples using Qiagen RNeasy kit (Qiagen Inc., Toronto, ON, Canada) as per the manufacturer’s instructions. The RNA concentration and purity was estimated spectrophotometrically on NanoDrop ND-100 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

All the RNA samples included in the study passed the quality control requirements (as assessed by the RNA integrity number or the OD 260/280 ratio) of the platform. Digital multiplexed NanoString nCounter analysis system (NanoString Technologies, Seattle, WA, USA)-based gene expression profiling was performed on 100 ng total RNA from each sample as input material according to the manufacturer’s instructions at the Ontario Genomics Institute, Toronto. Tumour RNA samples were subjected to analysis by nCounter Human inflammation panel consisting of 184 human inflammation genes with 6 six housekeeping controls (Supplementary Table 1) in the pre-built panel (NanoString Technologies). In this assay, single-target transcripts in the reaction are represented in the form of colour-coded barcodes. The resulting material was incorporated into an overnight hybridisation reaction, carried out by combining 5 μl of the total RNA sample with 20 μl of nCounter Reporter probes in hybridisation buffer and 5 μl of nCounter Capture probes for a total reaction volume of 30 μl. The hybridisations were incubated at 65°C for ~16–20 h. During the overnight hybridisation reaction, probe pairs are present in large excess to ensure that each target finds a probe pair. Abundances of specific target molecules can then be quantified using the nCounter Digital Analyzer to count the individual fluorescent barcodes and assess target molecules present in each sample. For each assay, a high-density scan (encompassing 600 fields of view) was performed. Following hybridisation, the cartridges were analysed in the Digital Analyzer that counts (that represent the number of molecules counted) the barcodes and further tabulates them.
NanoString data analysis. Normalisation of raw data was performed using the nSolver software (NanoString Technologies) as we previously reported (Martin et al., 2014). The raw NanoString counts were initially subjected to normalisation for all target RNAs in all samples based on built-in positive controls. This step accounts for intersample, experimental variation such as hybridisation efficiency and post-hybridisation processing. The geometric mean of each of the controls is calculated that indicates the overall assay efficiency. The housekeeping genes are then used for mRNA content normalisation. To facilitate downstream statistical analysis, values of < 0 were blanked and considered equal to 1. Following the initial normalisation steps, data were imported to Graphpad Prism software (GraphPad Software, Inc., La Jolla, CA, USA) for statistical analysis. Multiple t-tests were performed with correction for multiple comparisons using Sidak–Bonferroni method. An expression difference with a P-value of < 0.05 was considered statistically significant. Pearson’s correlation analysis was performed using Graphpad Prism software to derive correlation coefficient between the expression of STAT1 and CXCL10 genes.

Technical validation of NanoString data by real-time quantitative PCR (qRT–PCR) in discovery cohort. Total RNA was subjected to cDNA synthesis using RT² first-strand cDNA synthesis kit (Qiagen Inc.) as per the manufacturer’s instructions. Custom multiplexed PCR array (SABiosciences) consisting of CFL1, CREB1, CXCL10, STAT1, MAP3K7, MKNK1, RIPK1 and MYD88 gene targets was used to perform qRT–PCR on samples from the discovery cohort. The GAPDH and GUSB genes were included as housekeeping controls. The PCR array design also included pre-built reaction control and positive PCR control. All qRT–PCR experiments were performed in duplicates on LC-450 light cycler (Roche Diagnostics, Mississauga, ON, Canada). Data analysis was performed using relative quantification by the ΔΔCt method (Schmittgen and Livak, 2001; Koti et al., 2013). In this study, inflammatory gene expression profiling, by NanoString technology, of HGSCs that were clinically classified as chemotherapy resistant and sensitive displayed significant (P < 0.05) different expression levels in eleven genes. STAT1, CXCL10, CREB1, MKNK1, MAP3K7, CFL1, PTK2, RIPK1, MYD88, CCL8 and CCL7 were overexpressed in the sensitive cohort compared with the resistant (Figure 1), consistent with the rationale that these chemokines and immune factors could be active in the host microenvironment and promote a more favourable drug response. To determine the reproducibility of these findings, technical validation of 8 of the 11 genes was performed using qRT–PCR. Technical validation of the differentially expressed genes showed full concordance with the Nano-String-based gene expression findings (Supplementary Figure 1). Of these eight genes more highly expressed in the sensitive cohort, the interferon (IFN)-inducible protein (IP-10)/CXCL10 is one of the

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Table 1. Clinicopathological details of high-grade serous ovarian cancer patients in validation cohort

| Age          | Low STAT1, n (%) | High STAT1, n (%) | Overall, n (%) |
|--------------|-----------------|------------------|---------------|
| N            | 88              | 95               | 183           |
| Mean         | 60              | 62               | 61            |
| Median       | 60              | 63               | 62            |
| Min          | 11.0            | 9.6              | 10.4          |
| Max          | 82              | 81               | 82            |
| FIGO         |                 |                  |               |
| I/II         | 76 (86.4%)      | 74 (77.9%)       | 150 (82%)     |
| Residual disease |               |                  |               |
| Unknown      | 15 (17%)        | 9 (9.5%)         | 24 (13.1%)    |
| ≤1 cm        | 37 (42%)        | 47 (49.5%)       | 83 (45.4%)    |
| >1 cm        | 36 (40.9%)      | 39 (41.1%)       | 76 (41.5%)    |
| Treatment    |                 |                  |               |
| Platinum/taxane | 70 (79.5%)   | 85 (89.5%)       | 155 (84.5%)   |
| Platinum/taxane + additional agent* | 4 (4.5%) | 7 (7.4%) | 11 (6%) |
| Platinum      | 2 (2.3%)        | 1 (1%)           | 3 (1.6%)      |
| Taxane        | 1 (1.1%)        | 1 (1%)           | 2 (1.1%)      |
| Cyclophosphamide/ carboplatin | 5 (5.7%) | 1 (1%) | 6 (3.3%) |
| Cisplatin/topotecan | 6 (6.8%) | 0 | 6 (3.3%) |

**Abbreviations.** FIGO = International Federation of Gynaecology and Obstetrics; STAT1 = signal transducer and activator of transcription 1; Clinicopathological details of the high-grade serous ovarian cancer (HGSC) cohort of 183 patients used for independent validation of STAT1 expression by immunohistochemistry. Additional agent: placebo or ganitumab (AMG479) or nintedanib (BIBF1120) or bevacizumab.
major targets of STAT1 activation by IFNγ. We therefore performed Pearson’s correlation analysis comparing the expressions of these two genes in the resistant (Figure 2A) and sensitive (Figure 2B) tumours to determine their respective correlation coefficients. This analysis revealed a strong positive correlation between the expression of STAT1 and CXCL10 genes within the resistant and sensitive groups with $r^2 = 0.81$ ($P < 0.001$) and $r^2 = 0.77$ ($P < 0.0002$), respectively. Moreover, the greatly increased expression levels of STAT1 in the sensitive tumours is consistent with this protein having a functional role in mediating an improved response to chemotherapy, underscoring the importance of confirming these findings at the protein level.

A total of 183 tumours were available for STAT1 IHC staining analysis based on the same classification criteria used previously (Liu and Matulonis, 2006; Wang et al., 2012). Following antibody optimisation (Figure 3A) a semiquantitative score from 0 to 3 (Figure 3B) was utilised to determine the overall staining intensity for the STAT1 protein. Log-rank test analysis revealed a significant ($P = 0.02$) association between low STAT1 expression and decreased progression-free survival (Figure 4). These data provide additional evidence that elevated STAT1 expression together with other genes involved in the Th1 immune response may be investigated more extensively as biomarkers able to predict chemotherapy response in HGSC. Although a significant association between levels of STAT1 expression was noted upon comparison of FIGO stages I and II (Figure 5A), the difference in expression was not significant when stages III and IV were compared. Distinct STAT1 expression at the earlier stages I and II suggests that genotypic heterogeneity during tumour progression and/or the presence of a temporal element underlying the mechanism of cancer immunoediting may abrogate the need for differential STAT1 expression at later stages of the disease. Furthermore, Kaplan–Meier analysis also showed a trend, approaching significance, between decreased STAT1 expression and shorter overall survival compared with patients with longer overall survival (Figure 5B). Additional validation studies in larger cohorts are needed to confirm these findings.

**DISCUSSION**

Activation of both the adaptive and innate immune response to cancers are regulated by IFN (Schreiber et al., 2011). Indeed, the antiproliferative roles of IFN in cancer are well established and have led to novel therapies across various cancers (George et al., 2012). The STAT1 signaling is essential to all three types of IFN pathways and therefore plays an important role in immunosurveillance (Tymoszuk et al., 2014). Specifically, IFN-induced STAT1 activation leads to its binding either to the interferon response elements or Gamma-activated sites, further activating the interferon-induced genes (Leitner et al., 2014). Studies in mice with defective STAT1 activation have shown increased tumour incidence attributed to its tumour-suppressive role in breast tumourigenesis (Levy and Gilliland, 2000; Koromilas and Sexl, 2013). Moreover, in breast cancer tissue biopsies there was selective downregulation of STAT1 protein in tumour cells relative to the surrounding stroma when analysed by immunohistochemistry (Koromilas and Sexl, 2013). These data in breast cancer tissues, and the results reported herein in ovarian cancer, highlight the key role of the microenvironment for tumour development, and the need to perform single-cell resolution analysis in order to determine protein expression in distinct cellular compartments (Koti et al., unpublished). The STAT1 impacts its effects by various
mechanisms, such as shaping the immune cell infiltration by changes in expression within the immune compartment (Saha et al., 2010) or by a proliferation arrest because of changes in its expression within the tumour epithelium (Klover et al., 2010; Raven et al., 2011). The IFN-mediated activation of STAT1 also leads to expression of the angiostatic chemokine CXCL10 by multiple cell types including antigen-presenting dendritic cells, macrophages, T cells, fibroblasts and epithelial cells (Groom and Luster, 2011). It is well established that the CXCR3/CXCL10 chemotactic axis is key to trafficking and differentiation of effector Th1 CD4+ cells, NK and CD8+ cells within inflamed tissues (Groom and Luster, 2011; Groom et al., 2012). Furthermore, increased levels of CXCL10 and CCL5/RANTES are associated with enhanced CD8+ T cell infiltration in melanoma, colorectal and gastric cancers (Ohtani et al., 2009; Kunz et al., 1999; Muthuswamy et al., 2012). Our findings are consistent with recent studies of the immune microenvironment in breast cancer, where a similar trend of decreased CXCL10 and STAT1 expression was associated with relapse (Ascierto et al., 2012). Similar findings correlating increased STAT1 expression in pancreatic (Sun et al., 2014) and colorectal (Simpson et al., 2010) cancers with improved outcomes have been reported. Interestingly, a similar pattern of overexpression of these genes has been observed in other conditions exhibiting immune-mediated tissue destruction such as allograft rejection (Spivey et al., 2011), hepatitis C infection (Zeremski et al., 2008) and autoimmune diseases (Yoshida et al., 2012). However, it is likely that the magnitude of change in expression required for a tumour–host immunological response in cancer in a chemonaive environment might be quite different to the response needed in other immune-mediated disease conditions. It is noteworthy that activation of cytokine pathways in stromal and immune cells within some models of the chemotherapy-induced tumour microenvironment may be associated with resistance, rather than sensitivity to chemotherapy (Juntilla and de Sauvage, 2013). However, it is likely that the mechanisms underlying natural immunosurveillance may differ to those that are responsible for chemotherapy-induced response in which cell-autonomous cytokine pathways appear to be activated (Sistigu et al., 2014). As recently reviewed...
showing a trend, approaching significance ($P_d$) in disease stage in all patients of FIGO stage I and II. Indeed, in vitro and therefore it can be potentially be incorporated in future clinical trials following further validation in larger cohorts. Indeed, independent validation of STAT1 expression by IHC confirms its predictive role in HGSC. Decreased immune activation and its consequence following progression because of inadequate immune surveillance, thus microenvironment is incapable of exerting its effect in tumour cell death. An under reactive immune effect mediated by the immune cells. An under reactive immune microenvironment is incapable of exerting its effect in tumour cell progression because of inadequate immune surveillance, thus leading to immune escape by the tumour cells. It should be noted that the tumour cells’ intrinsic ability to modulate the secretion of cytokines and chemokines in the immediate vicinity of proliferating tumour could also provide cellular cross-talk that could contribute towards further modification of the local cellular immune/inflammatory microenvironment. Such a synergistic interaction between these compartments might be expected to lead to cellular variability in the expression levels of STAT1 in the epithelial and stromal elements. Although future longitudinal studies in this area need to be performed in order to correlate the decreased immune activation and its consequence following adjuvant chemotherapy, these initial associations provide insights into the design of novel immunomodulatory therapies that can be applied as adjuvant to current chemotherapy regimens in HGSC. The interferon pathway contributes significantly in modulating the tumour inflammatory microenvironment. Independent validation of STAT1 expression by IHC confirms its predictive role in HGSC and therefore it can be potentially be incorporated in future clinical trials following further validation in larger cohorts. Indeed, in vitro and in vivo studies are warranted to interrogate the mechanistic basis of its expression and its role in therapeutic response.

Overall, our findings provide the basis of mechanisms leading to tumour immune suppression in HGSC and warrant further investigation. In conclusion, the inflammatory gene signature suggestive of a pre-existing under reactive tumour immune microenvironment in chemotherapy-resistant HGSC patients must be explored further and taken into consideration in the design of novel adjuvant immunomodulatory therapies in HGSC.

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**CONFLICT OF INTEREST**

The authors declare no conflict of interest.

**AUTHOR CONTRIBUTIONS**

The study was conceived and designed by Madhuri Koti, Jeremy Squire and Anne-Marie Mes Masson. Sample preparation and NanoString data validation by qRT–PCR was performed by Madhuri Koti, Alex Siu and Mallikarjun Bidarimath. NanoString data analysis was performed by Kirsteen Mclean from NanoString.
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