Abstract

This study aims to identify reliable prognosis markers to predict patient outcome at surgery in high-grade serous epithelial ovarian cancer by a real-time quantitative PCR (RT-q-PCR)-based test. Seventeen tissue samples from serous epithelial ovarian cancer patients were screened by DNA microarray to identify genes differentially expressed between tumors from patients who relapsed within 18 months and tumors from patients showing no relapse or relapsed after 24 months after initial diagnosis. RNA expression of a subset of genes was validated by RT-q-PCR in the initial set of 17 samples. From these results, a refined list was selected and tested in independent samples from 41 serous tumors. Expression was associated with time to relapse and clinical variables. Microarray analysis identified a profile of 34 differentially expressed genes. RT-q-PCR validated the expression profile of a subset of seven genes in the initial set of patients. Differential gene expression was also validated in an independent set of patients. Low BTF4 or GCS expression was strongly associated with poor outcome in Kaplan-Meier analysis ($P < 0.05$, log-rank test) and Cox univariate as well as in multivariate analyses with a higher hazard ratio than clinical variables, such as residual disease, age, stage, and grade. (Cancer Epidemiol Biomarkers Prev 2008;17(4):913–20)

Introduction

Epithelial ovarian cancer (EOC) is the fourth leading cause of cancer-related death in women and represents the most lethal gynecologic malignancy (1). Due to its lack of symptoms, this disease is diagnosed at an advanced stage (stage III or IV) when the cancer has already spread to secondary sites. The standard treatment for these patients is surgery and platinum-based chemotherapy, although the disease often progresses even after surgery and becomes resistant to standard chemotherapy in less than 2 years (2). Consequently, the survival rate of patients with advanced-stage EOC is extremely low (<40%). For patients with invasive EOC, aggressive treatment, such as i.p. chemotherapy, is more effective, but due to the toxicity, patient stratification is an important variable when choosing such therapeutics options (3). Up to now, there are no reliable clinical factors that can properly stratify patients who would be best suited for aggressive first-line chemotherapy. Clinical variables, such as disease stage and residual disease, are helpful in the management of patients after their surgery to establish the first line of treatment (4) but are inefficient for those with advanced-stage disease. Accordingly, reliable markers independent and complementary to clinical variables are needed for a better management of these patients.

For several years, efforts to identify reliable prognostic factors have focused on molecular markers. A large number of molecular markers have been investigated to date, usually by immunohistochemistry, but specificity, sensitivity, and reproducibility are often lacking. Therefore, their clinical usefulness remains uncertain and still needs to be validated. More recently, genomic and proteomic analyses have emerged as powerful tools for identifying prognostic cancer markers. A large number of promising candidates have been identified by these techniques for cancers of different origin, such as breast, prostate, melanoma, B-cell lymphoma (5-9), and ovary (10, 11). These semiquantitative methodologies have shown low reproducibility on different platforms, indicating that they may be difficult to implement as clinical tests. Nevertheless, genomic and proteomic analyses are helpful and rapid screening tools for the high-throughput identification of potential markers. With more quantitative, reliable, and standardized techniques, such as real-time quantitative PCR (RT-q-PCR) for the measurement of RNA levels and ELISA for the measurement of protein levels, candidate genes can be validated.
and tested for their clinical utility. In contrast to ELISA, RT-q-PCR is not dependent on antibody availability and sensitivity and thus may facilitate the initial validation and eventual use of a greater number of markers.

In this study, we identified clinically relevant RNA prognosis markers that could be applied in a molecular prognosis test using a RT-q-PCR–based assay. As a first step, we screened EOC tumor RNA for potential markers using Affymetrix-based gene expression microarray platform. Because the disease often progresses and becomes resistant to standard chemotherapy in less than 2 years (2) and because 21 months is the median disease-free survival (DFS) after diagnosis in advanced-stage EOC patients, we defined two groups of tumors according to the progression-free interval, where tumors from patients who relapsed within 18 months after surgery corresponding to 13 months after chemotherapy treatment formed one group and tumors from patients who did not relapse or relapsed 24 months after surgery or chemotherapy formed a second group. In a second step, we applied RT-q-PCR to test a subset of these markers in two different groups of serous tumors. Finally, Kaplan-Meier and Cox regression models were used to assess association with patient survival. Sensitivity and specificity of the markers were also tested.

Materials and Methods

Patients and Tissue Specimens. Tumor samples from 177 patients were collected and banked in liquid nitrogen following appropriate consent from patients undergoing surgery within the Division of Gynecologic Oncology at the Centre Hospitalier de l’Université de Montréal from 1995 to 2004. An independent pathologist scored tumor grade and a gynecologic oncologist scored tumor stage and residual disease according to criteria from the International Federation of Gynecologists and Obstetricians (12). Clinical data on survival and progression-free interval were defined according to Response Evaluation Criteria in Solid Tumors criteria (13). Patient survival was calculated from the time of diagnosis until the first progression. For the microarray study, RNA was purified from samples collected between 1995 and 2002. The majority of samples were excluded based on inappropriate histopathology, incomplete follow-up, preoperative chemotherapy, or insufficient material. Less than 10% were excluded on RNA quality, and this was not correlated to age of sample. RNAs used for hybridization to the Affymetrix HuFL arrays were selected based on sufficient quantity and a RNA integrity number score of >8.

In total, 17 samples matched the eligibility criteria for this study. For the RT-q-PCR, 40 independent patients were included based on RNA quality and eligibility criteria. Eligibility criteria for inclusion in the study were as follows: no preoperative treatment, tumors of grade 2 or 3, clinical follow-up of at least 18 mo or until death; and completed informed consent. All patients received a Taxol/Carboplatin chemotherapy as an initial therapy after surgery. A single gynecologic oncologist reviewed the clinical data for all patients. The characteristics of the tumors and patient outcome for the sample sets are summarized in Table 1.

<table>
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<th>Sample properties</th>
<th>No. samples</th>
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<td>&lt;2</td>
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<td>Mean survival (mo)</td>
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*U test was used to compare the distribution of samples in both sets. No statistical difference was shown (P > 0.05).

RNA Extraction and Microarray Analysis. Total RNA was extracted from homogenized tumor tissue with...
Trizol reagent (Life Technologies, Inc.). Good quality was assessed with a 2100 Bioanalyzer with a RNA 6000 Nano LabChip kit (Agilent Technology). Affymetrix HuFL arrays were used to hybridize label targets prepared from total RNA. Hybridization assays and data collection were undertaken at the McGill University and Genome Quebec Innovation Centre (Montreal, Quebec, Canada) as described in the Web site. Affymetrix raw values were assigned by Affymetrix GeneChip software (MAS4) with an accompanying reliability score of present (P), marginal (M), and ambiguous (A). No genechip used in this study had >30% A score. Presence of stromal cell was estimated by detectable expression of CD31 and myosin in the GeneChip (probes D10667, L34657, and X96783). No genechip used in this study had detectable signal for these probe sets. Global normalization and preprocessing of the data were previously described in detail (14, 15). Data sets are available online on publication. Candidate genes that exhibited statistically significant differences in expression within a tested set were selected using the significance analysis of microarray (SAM) (10) (1,000 permutations done and false discovery rate of <5%; ref. 16) and the Mann-Whitney U test with the GeneSpring software (Agilent Technology; P < 0.05 with Benjamini and Hochberg false discovery rate of 5%).

Real-time Quantitative PCR. cDNA synthesis was done using the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen Life Technologies) according to the manufacturer’s protocol. Q-PCR was done with a Quantitect SYBR Green PCR reagent as described by the manufacturer’s instructions (Qiagen, Inc.) using a RotorGene 3000 Real-time Centrifugal DNA Amplification System (Corbett Research, Montreal Biotech, Inc.). Q-PCR analysis for each gene was done in duplicate in two independent analyses but when two experiments were concordant, and where the average just failed to reach significance, a third experiment was done and used to determine the average. The Pfaffl method served to evaluate the relative quantity of gene expression with the appropriate internal control (see also Supplementary Table S1 for primer sequences; ref. 17).

Statistical Analysis. Differential expressions of candidate genes measured by RT-q-PCR were evaluated by a U test. For survival and progression analyses, we used the Cox survival model with time-dependent covariate and Kaplan-Meier curves coupled with the log-rank test. Receiver operating characteristics curves were charted for each marker to define a threshold of expression corresponding to the best sensitivity and specificity for patient progression before or after 18 mo from initial diagnosis (P < 0.05 and area >0.70). For Cox regression analysis, markers were treated as categorical variables based on the threshold of expression. Survival and progression analyses done using time after the cessation of the first treatment of chemotherapy as the starting point were also done (see Supplementary Fig. S2 and Supplementary Table S3). All statistical analyses were done using Statistical Package for the Social Sciences software version 11.0 (SPSS, Inc.), and statistical significance was set at P < 0.05.

Results

Candidate Genes Related to Disease Progression. To identify potential genes whose expression could be used as prognosis markers, a supervised microarray analysis was done using expression profiles of 17 serous tumors that were obtained from 10 patients showing early disease progression (within 18 months after surgery) and 7 patients who showed no disease progression in the 2 years following diagnosis. A total of 34 probe sets identified differential expression between these two groups (Fig. 1) with six common genes based on two statistical analyses (see Materials and Methods). Because an objective prognosis of ovarian patients based on a limited set of genes would be more appropriate for clinical application, we refined this analysis to the only six candidate genes identified by both statistical methods (GCS/GCLC, CMHE1/HLA-E/HLA-6.2, HLA-C, BTF4/BTN3A.2, FUCA1, and SSX2) and to an additional three genes that presented a 2-fold difference in each of the two analyses (C1r/CTS, PTP4A2/PTF1/CAAX2)/PRL2/HH12/HH7-2, and YMP/EMP3). Because the probe set for HLA-C no longer corresponds to the correct Genbank number, we eliminated this gene from further analysis.

We validated the microarray results by RT-q-PCR on RNA corresponding to the 17 samples included in our microarray analysis. Statistically significant (P < 0.05, U test) overexpression of BTF4, CMHE1, PTP4A2, YMP, C1r, and GCS was observed between the two groups (Fig. 2). SSX2 showed expression in only two samples and no FUCA1 expression was detectable by RT-q-PCR. Only the six genes presenting significant differential expression or a trend toward significance were chosen for further analysis.

Independent Validation of Candidate Genes on a Larger Set of Serous Samples and Association with Survival. To determine whether the six candidate RNA markers identified by microarray and validated by RT-q-PCR may be clinically relevant and reliable to define the prognosis, we tested RNA expression on a large independent set of 41 serous samples. This test set contained samples from 41 patients, of which 26 were associated with early disease progression and 14 with late disease progression (Table 1). Among the candidates tested, only BTF4 (P = 0.0001, U test) showed significant differential expression between the two patient groups (Fig. 3), whereas a strong trend toward significance was observed for GCS (P = 0.005, U test).

Kaplan-Meier analysis and the Cox proportional hazard model were used to estimate the association between BTF4 or GCS expression and DFS or overall survival (OS) for the 41 patients with serous tumors. The optimal threshold values that could be used for each marker to predict survival and assign labels of early or late disease progression risk were estimated using receiver operating characteristic curves. There was a strong association between BTF4 expression and DFS (P = 0.0001, log-rank test) or OS (P = 0.01, log-rank test; Fig 3; Table 2). Mean DFS and OS were 54 and 64 months, respectively, for patients with high levels of BTF4
expression compared with 11 and 37 months, respectively, for patients with low levels of BTF4 expression. GCS expression profile also showed a significant association with DFS and OS (P = 0.04 and 0.01, log-rank test, respectively) but was weaker than that of BTF4 (Table 2).

In univariate and multivariate Cox regression analyses, several clinical prognostic factors, such as residual disease, stage, grade, and age, were evaluated, in relation to BTF4 and GCS expression. Low BTF4 expression showed the highest hazard ratio (HR) for DFS (HR, 0.195; 95% confidence interval, 0.079-0.079; P = 0.0001), outdoing all other variables. Low GCS expression showed the highest hazard risk for OS (HR, 0.181; 95% confidence interval, 0.03-0.03; P = 0.03; Table 2). In multivariate analysis, only BTF4 remained an independent variable of prediction with a high risk of progression (HR, 0.143; 95% confidence interval, 0.488-0.488; P = 0.001), whereas both BTF4 and GCS remained independent variables for prediction of death (HR, 0.189; 95% confidence interval, 0.840-0.061; P = 0.03 and HR, 0.178; 95% confidence interval, 0.826-0.04; P = 0.03, respectively; Table 2).

Performance of BTF4 and GCS in Patient Survival Prediction. To determine the clinical performance of BTF4 and GCS gene expression to predict the DFS and OS in our serous EOC cohort, the sensitivity and specificity of both candidates were evaluated using the label assigned previously from the receiver operating characteristic analysis. Sensitivity for DFS (the fraction of patients correctly diagnosed with EOC progressing within 18 months after diagnosis) was 77% (n = 20 of 26) for BTF4 and 85% (n = 22 of 26) for GCS. Specificity for DFS (the fraction of patients correctly diagnosed with no disease progression within 18 months after surgery) was 87% (n = 13 of 15) and 73% (11 of 15) for BTF4 and GCS, respectively. Two independent experiments estimated the reproducibility of the test at 96% for BTF4 and 85% for GCS. In a reproducible way in two independent experiments, three patients relapsing early and two patients relapsing late were misclassified. The combination of two or more candidates identified in this study did not improve the prognostic value of BTF4 (data not shown). The efficiency of the markers to predict OS was lower than for DFS. BTF4 showed 61% (n = 17 of 28) sensitivity and 77% specificity (n = 10 of 13).

To evaluate the clinical potential of the markers, we also determined their variability of expression within independent EOC samples obtained from initial surgeries from the same patients. For nine patients, of whom five had early disease progression and four had late progression, two or three samples were available from either omentum or right or left ovaries. Among these nine patients, the BTF4 marker showed a high concordance in prognostic prediction, with only a single contradictory assignment in one patient, and this result was reproducible (analysis conducted in two independent assays). However, this patient was also the only one among the nine with early disease progression (12 months) and that presented a long-term survival (4 years). In contrast, the GCS marker was less robust, as independent samples assigned three of nine patients to separate prognostic outcomes, suggesting that GCS is more physiologically variable and/or more sensitive to the source of sampling than BTF4.

Discussion

Our study showed that combined DNA microarray and RT-q-PCR identified quantifiable molecular markers to distinguish between serous EOC patients who relapse within 18 months from those who will relapse later than 2 years after initial surgery. This approach offers several advantages. First, it avoided technical bias because we used a second technique to validate the initial results obtained with the microarray. Second, it allowed us to test the robustness of candidates as proven in an...
independent patient cohort. Third, RT-q-PCR has proven to be a quantitative and reproducible technique. Fourth, RNA detection is not dependent on antibody availability, in contrast to the quantitative technique based on protein detection, such as ELISA. Even if RNA is less stable and more easily degraded than protein, sufficient care of quality and standardization during the entire sampling process can counteract this disadvantage.

The analysis presented here reveals interesting gene expression profiles (Fig. 1; Table 2). Comparison with previously published studies (10) is difficult because there does not as yet exist a recognized standard that defines good and bad prognosis. In our study, several of the selected genes are related to the MHC and immunologic response, with significantly lower expression in the patient group who progresses rapidly (Fig. 1). It is tempting to speculate that escape from an immune response is a major mechanism in EOC progression as it becomes more aggressive. This hypothesis is in line with recent observations where the presence of intratumoral infiltrating T cells in ovarian tissues is associated with a longer patient survival (18) and immunosuppressive condition in EOC tissues is associated with a shorter patient survival (19). In our study, among the prognostic molecular markers identified, we found BTF4/BTN3.A2, a protein that belongs to the BT3/butyrophilin family, a B7 subfamily, which is also a subclass of the immunoglobulin superfamily. BT3 molecules are expressed on endothelial cells (20, 21), in the membrane layer surrounding milk fat-secreting droplet from mammary epithelial cells (22), on immune cells and tumor cell lines (23). Although the mRNA expression of BTF4 in EOC tissues is likely to be due to epithelial tumor cells, because it is also detected in EOC cell lines, we cannot exclude the possibility that BTF4 expression is also due in part to infiltrating immune cells such as lymphocytes. These molecules are suspected to be involved in the antitumoral immune response because cytokines, such as IFN-γ and TNF-α, known to stimulate the antitumoral response, can also up-regulate the expression of BT3 molecules (23). BTF4 is also part of an emerging list of autosomal genes exhibiting allelic expression (24). The notion that allelic expression may also be present in EOC tissues is reinforced by the observation that two EOC cell lines, heterozygous for BTF4, exhibited deviations from the expected 50:50 allele ratio in the analysis of gene expression (Supplementary Table S2). Further exploration of BTF4 expression in EOC disease is warranted to determine if differential allelic expression is also predictive of early relapse.

The second candidate, GCS/GCLC, the catalytic subunit of glutamylcysteine synthetase, also known as glutamatedisulfide ligase, is a key enzyme in glutathione biosynthesis, an important antioxidant participating in the detoxification reactions of several drugs (25, 26). Through several of its functions, glutathione plays an important role in protecting cellular components from the cytotoxic effect of drugs. Several in vitro studies have shown...

**Figure 2.** RT-q-PCR validation of microarray analysis. Black, tumors used in the microarray analysis derived from 7 patients who relapse after 24 mo; gray, tumors used in the microarray analysis derived from 10 patients who relapse within 18 mo after initial diagnosis. Columns, mean results of two independent experiments in duplicate; bars, SE. Relative fold change was calculated according to the Pfaffl algorithm using ERK1 as an internal control gene and TOV1054D was arbitrary chosen as the reference sample. Statistical analysis was done by the Mann-Whitney U test.
shown that inhibition of GCS enhanced the toxic effect of chemotherapeutic agents (27-29), although this contrasts with our observation that high GCS expression is related to a better prognosis. However, the subunit GCLC is less effective than the regulatory subunit GCLM to induce drug resistance in adenocarcinoma cells (30, 31). More research would be required to understand the role of GCS in chemotherapy response of ovarian cancer patients (32, 33).

The most important aspect of this study is the discovery of new prognostic factors for EOC that could be easily transposed as a quantitative clinical test even if the initial cohort used for DNA microarray screening was relatively small (17 samples). As of the initial sample size used was small, it is possible that some candidates were statistically identified by chance. In addition, in this initial cohort, the group of patients with a late relapse included four samples of early-stage disease that may have generated a bias. This limitation was overcome by validating the candidates in an independent set of tumors and may explain why only two of six candidate genes were statistically validated on the second set of patients. Among the two validated markers, BTF4 seems to be the most interesting. Not only did it have a prognostic significance in univariate and multivariate analyses, but it also showed the highest HR compared with the other molecular and clinical prognostic variables tested.

Even if BTF4 marker reaches 77% sensitivity and 93% specificity and is a competitive candidate compared with the other markers and clinical variables identified to date, it would be necessary to increase its efficiency ideally to 99%. In total, 4 of 41 patients were misclassified and these differences could not be correlated.

Figure 3. Analysis on 41 invasive serous tumors and association with patient survival. Black, serous tumors derived from 15 patients who did not relapse or who did after 24 mo; gray, serous tumors derived from 26 patients who relapsed within 18 months. Relative fold change was calculated according to the Pfaffl algorithm using ERK1 as an internal control gene and TOV1054D was arbitrary chosen as the reference sample. Mean results of two independent experiments in duplicate are presented. A and B. Statistical analysis was done using Mann-Whitney U test. Kaplan-Meier DFS (C) and OS (D) curves in 41 patients with serous EOC. Significance (P) is calculated by log-rank test.
with any apparent clinical feature (data not shown). Further investigations are needed to address this issue, which may be related to the high heterogeneity of ovarian cancer (34). This result also underscores the probable need for combining several prognostic markers to attain sufficient specificity for an eventual clinical application. Combination with other independent molecular markers could improve the clinical performance of BTF4, as to date no individual marker has shown sufficient sensitivity and specificity, and we have previously shown the power of this approach in EOC disease in the analysis of serum markers initially identified by gene expression microarray analyses (35). As the expression of the individual markers identified in this study was highly correlated, they did not act as independent prognostic markers in a multivariate analysis and thus could not be used in combination to improve specificity or sensitivity. The similarity in expression patterns may be related to the fact that several of the identified candidate genes are localized to the small arm of chromosome 6 and may point to a role of this chromosomal region in the ovarian cancer progression. Whether BTF4 and/or GCS can act as independent markers with other candidates identified in the literature remains to be investigated.

In conclusion, our study presents initial results identifying potential clinically interesting RNA molecular markers of disease progression in EOC. However, it is still critical to further evaluate these markers in a larger patient cohort to better evaluate the potential of these markers and to understand their relation to the whole spectrum of ovarian cancer. We showed the advantage of coupling a high-throughput technique as a screening tool for the identification of potential candidates with a quantifiable technique of marker validation and testing. Using this strategy, we have highlighted the potential of BTF4 as a prognostic marker and showed its power compared with clinical factors commonly used to date.

Acknowledgments

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BTF4/BTNA3.2 and GCS as Candidate mRNA Prognostic
Markers in Epithelial Ovarian Cancer

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