Differential gene expression analysis of ovarian cancer in a population isolate

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Summary

Gene expression products represent candidate biomarkers with the potential for early screening and therapy of patients with ovarian serous carcinoma. The present study, using patients that originate from the population isolate of South Tyrol, Italy, substantiates the feasibility of differential gene expression analysis in a genetically isolated population for the identification of potential markers of ovarian cancer. Gene expression profiles of fresh-frozen ovarian serous papillary carcinoma samples were analyzed and compared to normal ovarian control tissues using oligonucleotide microarrays complementary to 14,500 human genes. Supervised analysis of gene expression profiling data identified 225 genes that are down-regulated and 635 that are up-regulated in malignant compared to normal ovarian tissues. Class-prediction analysis identified 40 differentially expressed genes for further investigation as potential classifiers for ovarian cancer, including 20 novel candidates. Our findings provide a glimpse into the potential of population isolate genomics in oncological research.

Key words: Gene expression analysis; Microarray; Ovarian cancer; Molecular marker; Population isolate.

Introduction

Ovarian carcinoma is the most lethal gynaecological malignancy in North American and Western European women. Contributing to the poor prognosis are the preponderance of late-stage disease at diagnosis, the frequent development of drug resistance, and the lack of reliable markers for diagnosis, prognosis, and therapy [1, 2]. Ovarian carcinoma is not a single disease, but a group of heterogeneous neoplasms which derive mainly from the ovarian surface epithelium. The most common histological subtypes are: serous, mucinous, endometrioid, clear cell, transitional cell, and undifferentiated [3]. Previous studies have identified genes related to ovarian carcinoma, including p53, c-myc, c-erb-B2, and K-ras, but none of them can reliably be employed as diagnostic or prognostic markers [4]. The only validated molecular marker in ovarian cancer is CA125, a large glycoprotein of unknown function, which is expressed in over 80% of ovarian cancers [5]. Even though changes in CA125 levels correlate to the clinical course of the disease and can be used to predict tumor progression and response to therapy, it has serious limitations as a diagnostic or prognostic tool [6]. The identification of potential tumor markers is urgently needed for a better understanding of the underlying biochemical mechanisms and regulatory pathways involved in ovarian tumorigenesis. The key technology for the study of the vast amount of genetic data is the DNA microarray, which has established itself as an indispensable research tool for biological and medical research. The main benefit of DNA microarrays is that they allow the investigation of differential gene expression of several thousands of genes within two independent samples in a comprehensive manner. The comparison of the expression and mutation profiles obtained from tumor cells and healthy cells enables us to gain new insights into the complexities of cancer without detailed previous knowledge [7, 8].

In the present investigation we have compared the transcriptomes of ovarian epithelial tumors and adjacent healthy tissue from South Tyrolean cancer patients. The province of South Tyrol in Northern Italy is a cultural-linguistic island with a genetically relatively homogeneous population and a highly developed health system [9]. Owing to reduction in genetic heterogeneity, isolated population groups are considered highly valuable for studying disease genes and mutations, and their interaction with environmental and clinical factors [10]. Previous studies have shown that novel cancer-related genes can be successfully identified through the analysis of isolated populations [10, 11]. Here, we report the identification of 860 genes that are differentially expressed in ovarian tumor samples compared to normal ovarian tissue using the Affymetrix GeneChip® microarray technology. Our specific focus has been on 40 genes which we iden-

Revised manuscript accepted for publication October 8, 2007

tified as classifiers of tumorous versus normal tissue through class prediction and hierarchical clustering. This study suggests that the analysis of differential gene expression of ovarian cancer can be successfully applied in a population isolate such as South Tyrol to expand our knowledge of the underlying biology of cancer.

Materials and Methods

Sample collection

For this study, we established a competence network across four regional hospitals of South Tyrol (Bolzano, Merano, Brunico, and Bressanone) and developed a full study protocol which was then approved by the local ethics committee of the Autonomous Province of Bolzano. Each study participant signed an informed consent. For each case in this study, an epithelial serous ovarian cancer sample and adjacent normal tissue from the same subject were collected. The sample type, the tumor histology, the percentage of tumorous cells and the percentage of necrotic cells in the tumor sample are indicated in Table 1. Normal ovarian tissue samples 6_N, 7_N and 8_N that have been included in this study as controls were obtained from a different source through an EC-approved collaboration with a clinical department.

Table 1. — *Samples used in this study and tumor sample classification (n.a. = not assessed).*

Sample Type	Sample Code	Histology type	Grading	Necrotic cells	Tumor cells
Normal	1_N			n.a.	n.a.
Normal	2_N			n.a.	n.a.
Normal	3_N			n.a.	n.a.
Normal	4_N			n.a.	n.a.
Normal	5_N			n.a.	n.a.
Normal	6_N			n.a.	n.a.
Normal	7_N			n.a.	n.a.
Normal	8_N			n.a.	n.a.
Tumor	1_T	Serous cystadenocarcinoma, partly papilliferous and partly solid	G3	–	70%
Tumor	2_T	Serous papilliferous cystadenocarcinoma	G2	10%	76%
Tumor	3_T	Serous papilliferous ovarian carcinoma	G2	1%	90%
Tumor	4_T	Serous papilliferous cystadenocarcinoma	G3/4	15%	80%
Tumor	5_T	Serous papilliferous adenocarcinoma, poor differentiation, mainly solid	G3-4	5%	80%

Gene expression profiling

Total RNA was extracted using TRIzol® reagent (InVitrogen) and purified using the RNeasy® Mini Kit (QIAGEN). RNA integrity was assessed using the Agilent® 2100 bioanalyzer and the RNA Nano LabChip® Kit (Agilent Technologies). For cRNA probe preparation, 8 μg of total RNA was linearly amplified using the One-Cycle Target Labeling Assay according to the manufacturer's instructions (Affymetrix, Santa Clara, CA); 15 μg of fragmented cRNA were hybridised on GeneChip® Human Genome U133A array (Affymetrix®) after quality checking on GeneChip Test3 array (Affymetrix). Standard Affymetrix procedures were applied for quality assessment.

Microarray data analysis

Data handling was mainly done using the Bioconductor Affy package [12]. Probe set intensities were computed using the GCRMA method and loess normalization. Probe sets that did not show broad interquantile intensity ranges within the experimental samples were filtered out by applying the Interquantile (IQR) filtering procedure (IQR ≤ 0.5). The filtered data led to 8404 grade A probesets. Genes differentially expressed in tumor versus normal samples were identified using Significance Analysis of Microarray (SAM 2.1) software [13] (minimal fold change = 2; false discovery rate < 1). Multiclass classification was performed by Prediction Analysis of Microarrays (PAM) [14] on the 4055 probe sets that passed the IQR filter and which were expressed in all samples (intensity > 100 in non-log scale). To perform hierarchical clustering of the selected probe lists, Euclidean distance and linkage methods were respectively used as distance and linkage methods within Spotfire 8.1 software.

Gene functional annotations

The differentially expressed genes were annotated using the on-line tool "Database for Annotation, Visualization and Integrated Discovery" (DAVID). DAVID functional annotations are derived primarily from Entrez Gene at the National Centre for Biotechnology Information (NCBI).

Results

Within the competence network established across four South Tyrolean regional hospitals we developed an efficient workflow system for sample collection, tissue transport in RNAlater solution, and pathological analysis of the samples (Figure 1) .

Differential expression analysis using a two-class unpaired data test (SAM) was performed on the 8404 probe sets that passed the IQR filter, resulting in a small number (149) of probe sets that were down-regulated in the tumor samples compared to normal ovarian tissue. To investigate if these results were due to non homogeneity among the samples, hierarchical clustering was performed on the selected probe sets list. Three of the normal ovarian samples (1_N, 2_N and 4_N) clustered with the tumor samples instead of the other normal ovarian tissues, probably due to the presence of tumor

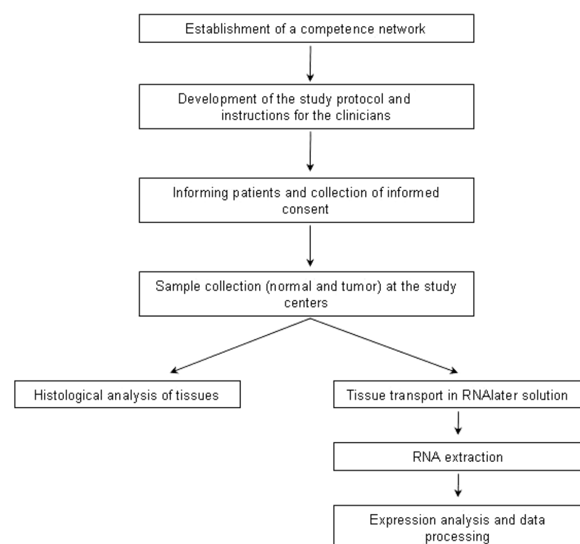

Figure 1. — Flow chart detailing the main processes involved in the competence net.

Table 2. — *Forty genes identified as potential class predictors. References to studies which have previously identified the gene as being differentially regulated in ovarian cancer are indicated in the last column.*

PAM rank (T = 2)	Gene	Gene title	SAM fold change	SAM up/ down	Ontology	OMIN	Ref
1	KLF4	Kruppel-like factor 4 (gut)	7.23913	down	transcriptional activator	602253	
2	AKAP12	A kinase (PRKA) anchor protein (gravin) 12	5.14236	down	anchoring protein	604698	
3	NR2F2	Nuclear receptor subfamily 2, group F, member 2	12.33229	down	transcriptional activator	107773	28
4	PBX3	Pre-B-cell leukemia transcription factor 3	6.5696	down	transcription factor activity	604698	23
5	PMP22	Peripheral myelin protein 22	3.95213	down	growth regulation	601097	4,23
7	DCN	Decorin	12.83013	down	extracellular matrix	125255	25, 26, 29
8	TNXB	Tenascin XB	4.65204	down	cell-matrix adhesion	600985	23
9	PRELP	Proline arginine-rich end leucine-rich repeat protein	3.58746	down	extracellular matrix	601914	
10	MYH11	Myosin, heavy polypeptide 11, smooth muscle	7.78747	down	muscle contraction	160745	23
11	KLF2	Kruppel-like factor 2 (lung)	7.33757	down	transcriptional activator	602016	30, 48
13	MAOA	Monoamine oxidase A	4.72467	down	amine oxidase activity	309850	
14	TCEAL4	Transcription elongation factor A (SII)-like 4	5.22394	down	unknown		27
15	GALNT6	UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 6	0.15933	up	O-linked glycosylation	605148	
17	LMOD1	Leiomodin 1 (smooth muscle)	3.74209	down	cytoskeleton	602715	23
18	C10orf56	Chromosome 10 open reading frame 56	3.96817	down	nucleic acid binding		
19	H3F3B	H3 histone, family 3B (H3.3B)	3.43818	down	chromosome organization and biogenesis	601058	
20	CD24	CD24 antigen (small cell lung carcinoma cluster 4 antigen)	0.1135	up	humoral immune response	600074	5, 21-24, 48
22	FXYD6	FXYD domain containing ion transport regulator 6	6.01794	down	ion transport	606683	
26	JUNB	Jun B proto-oncogene	3.60332	down	transcription factor	165161	45,48
27	ITM2A	Integral membrane protein 2A	19.20317	down	integral to membrane	300222	23, 25, 26
28	FHL2	Four and a half LIM domains 2	5.15755	down	nucleus	602633	
29	SERPINF1	Serine (or cysteine) proteinase inhibitor, clade F, member 1	5.03964	down	neurotrophic protein	172860	23
30	GSTM5	Glutathione S-transferase M5	6.7164	down	conjugation of reduced glutathione	138385	23, 46, 47
31	JAM3	Junctional adhesion molecule 3	4.90563	down	cell-cell adhesion	606871	
32	NR4A1	Nuclear receptor subfamily 4, group A, member 1	6.34201	down	ligand-dependent nuclear receptor activity	139139	23,48
34	ACTR2	ARP2 actin-related protein 2 homolog (yeast)	0.33249	up	Arp2/3 protein complex	604221	
38	KRT18	Keratin 18	0.05185	up	structural molecule activity	148070	4, 5, 23
41	VLDLR	Very low density lipoprotein receptor	4.91199	down	low-density lipoprotein receptor activity	192977	23
42	ADAMTS1	A disintegrin-like and metalloprotease (reprolysin type) with thrombospondin type 1 motif, 1	8.61058	down	metalloendopeptidase activity	605174	
43	CBX7	Chromobox homolog 7	4.93005	down	chromatin binding	608457	
45	SIDT2	SID1 transmembrane family, member 2	3.39928	down	unknown		
47	TSPAN13	Tetraspanin 13	0.20642	up	integral to plasma membrane		
50	GLTSCR2	Glioma tumor suppressor candidate region gene 2	3.98729	down	unknown	605691	
52	FLJ11200	Hypothetical protein FLJ11200	2.2273	down	unknown		
53	CPZ	Carboxypeptidase Z	3.43119	down	carboxypeptidase A activity	603105	23
55	UCP2	Uncoupling protein 2 (mitochondrial, proton carrier)	0.12386	up	transporter activity	601693	5
56	SPON1	Spondin 1, extracellular matrix protein	0.20832	up	cell adhesion protein	604989	15
57	NDRG2	NDRG family member 2	6.66087	down	cell differentiation	605272	
58	NBL1	Neuroblastoma, suppression of tumorigenicity 1	4.66712	down	negative regulation of cell cycle	600613	
59	ITGA6	Integrin, alpha 6	0.3626	up	cell-matrix adhesion	147556	

cells (Figure 2). We decided therefore to exclude the three samples from the differential expression analysis and to include as controls three external samples instead. SAM was run on the remaining samples and resulted in 1056 significantly differentially regulated probe sets (Figure 3). Of these: 268 probe sets (225 genes) were down-regulated in the tumor samples compared to the normal ovarian tissue, 788 probe sets (635 genes) were up-regulated in the ovarian cancer sample compared to the normal tissue. PAM was run for class prediction. Applying a threshold value of 2, 60 probe sets (54 genes) were calculated to be the more likely to be class predictor genes (Figure 2). Hierarchical clustering of the 60 probe sets identified with PAM is shown in Figure 3. These genes show a different pattern of expression in normal tissues compared to the cancer samples. However sample 4_N clustered with the tumors. The removal of this sample from the data did not improve the results. Of the 54 genes, we classified 40 genes as significant after combining both PAM and SAM analysis (Table 2).

Discussion

Over the last six years a number of studies on expression profiling of ovarian cancers have been published, but so far no reliable prognostic marker has been found and the molecular pathways involved in the initiation and progression of ovarian cancer are still poorly understood [15]. Cancer is a highly polymorphic disease resulting from the accumulation of genetic and epigenetic aberrations in interaction with environmental and clinical factors [16]. The importance of isolated populations for revealing the genetic etiology of common diseases, including cancer, has been highlighted in recent years [17, 18]. The advantages of studies on isolated populations are the more uniform genetic and environmental

Fig. 2

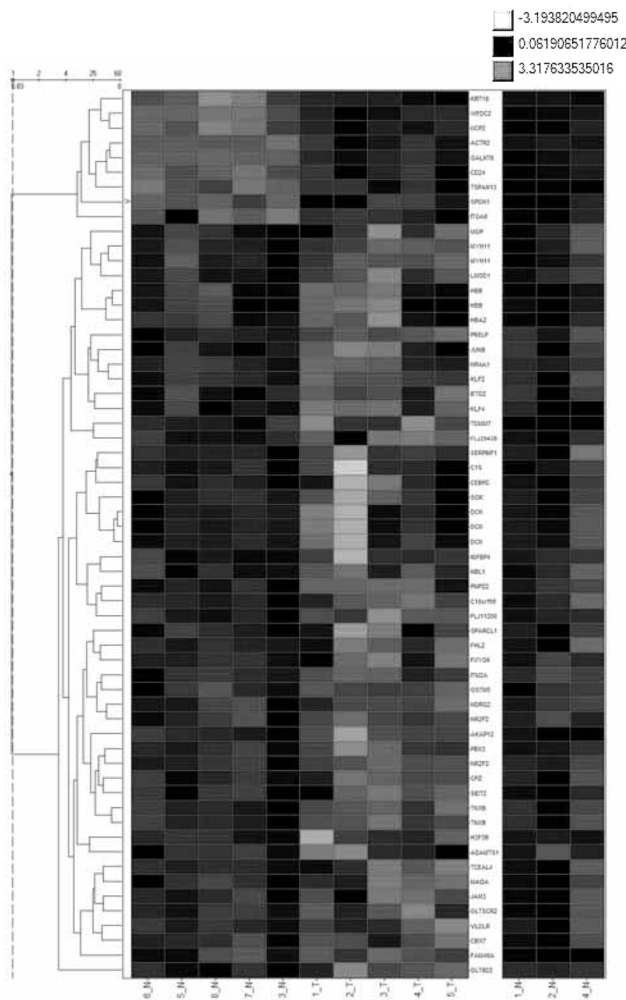

Fig. 3

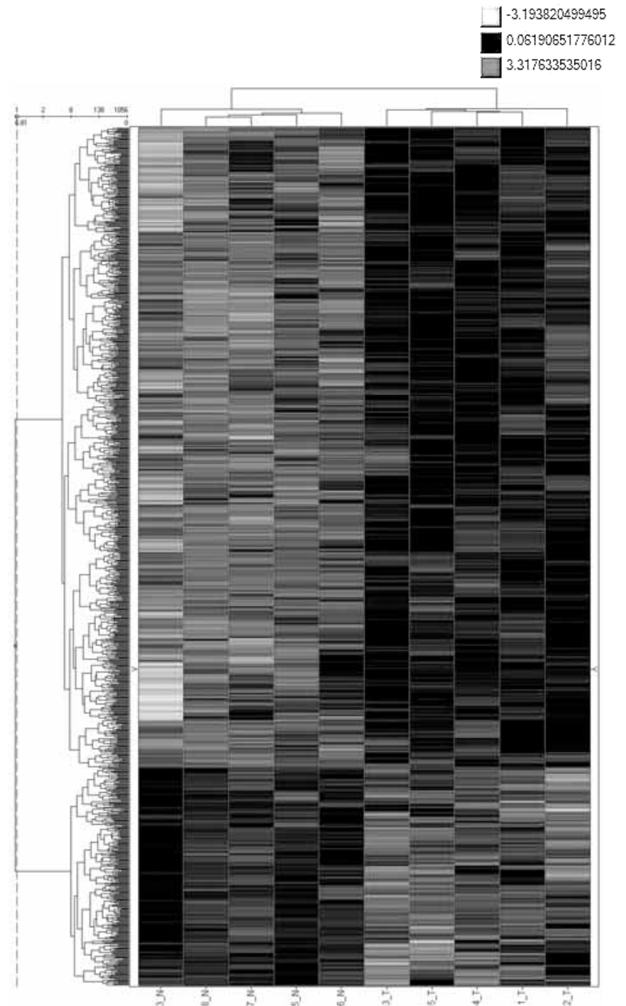

Figure 2. — Heat map of the expression profiles for the samples included in this study for the 60 probe sets identified by means of PAM. These were used as minimal set of genes sufficient to discriminate normal vs tumor tissues. Whereas most of the samples are correctly clustered together, three from the normal set (1_N, 2_N, 4_N) were classified as being more similar to cancer tissue rather than normal. These were not included in the differential expression analysis.

Figure 3. — Unsupervised hierarchical clustering of the 1056 probe sets filtered by SAM analysis in the selected samples. Each column represent a different sample while each row a different probe set. The cluster method used correctly identified the two sets of tissues (normal and tumor) and shows the blocks of genes which are up-regulated or down-regulated.

backgrounds which allow a more efficient approach to the research of cancer. South Tyrol, the northern-most province of Italy, represents a very good location for the study of several diseases, in many fields of genetic medicine [19, 20].

In the present study we have analyzed five epithelial serous ovarian cancer tissues and the corresponding normal ovarian tissue from well characterized South Tyrolean cancer patients by microarray analysis. It has been shown that the genetic background of unrelated individuals causes variance in tissue gene expression levels [21]. Gene-expression profiling in individuals within a genetic isolate such as South Tyrol can somewhat reduce such inter-sample variations, thus allowing the detection of significant pathology-related changes in gene expression with fewer samples. In our sample-set we identified 225 genes that are down-regulated and 635 that are up-regulated in tumors compared to normal ovarian tissue. Through class prediction and hierarchical clustering we identified 40 genes which may be related to molecular events involved in the genesis and development of ovarian cancer. These genes included common oncogenes and tumor suppressor genes with known roles in carcinogenesis, as well as genes with no known role in cancer. At least 20 genes have already been reported in previous studies of ovarian cancerogenesis, including CD24 (small cell lung carcinoma cluster 4 antigen) [22-25], ITM2A (integral membrane protein 2A) [24, 26, 27], keratin 18 [4, 5, 24], TCEAL4 [28], NR2F2 [29], decorin [27, 30], and KLF2 [31]. CD24 is known to be upregulated in hematological malignancies and different types of solid tumors, including ovarian cancer [5, 24]. Recent

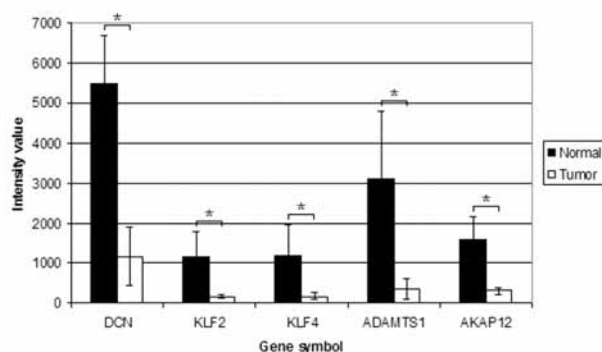

Figure 4. — Gene expression levels of five genes involved in the genesis and development of solid tumors in paired (tumor vs normal) ovarian tissues. Values are reported as mRNA expression levels, i.e. normalized fluorescent intensity. Each bar represents the average of single sample intensity values per population. Lines indicate standard deviations while * indicates whether the difference is significant between the two groups at $p < 0.05$ (Student's t-test).

studies have demonstrated that CD24 could act as a new independent prognostic marker for survival time of ovarian cancer patients [22, 25]. Previous gene expression analyses have revealed that the human Kruppel-like factors KLF2, KLF4, and KLF6, all play an important role in transcription modulation and cancer development [32, 33]. KLF2 is significantly down-regulated in ovarian cancers and its re-introduction leads to an inhibition of cell growth and increased DNA damage-induced apoptosis, making it a possible candidate for novel therapeutic strategies [31]. Another gene that we found to be down-regulated is the transcription elongation factor A (SII)-like 4 (TCEAL4), which has just recently been associated with the development of thyroid cancer [28]. TCEAL4 is expressed ubiquitously in human tissues and was found to be under-expressed in anaplastic thyroid cancer [45]. Decorin (DCN) is a small leucine-rich proteoglycan involved in cell proliferation and modulation of the extracellular matrix, and is suppressed in most tumor cell lines [27, 34, 35]. The cytostatic effects of decorin via inhibition of the transforming growth factor-ß (TGF-ß) have been shown in tumor cells independent of their origin [34, 36, 37]. These findings make decorin an interesting candidate for cancer therapy.

Altogether, 50% of the 40 genes identified in our analysis have been described previously in studies of ovarian cancer, providing some validity to our study. To our knowledge, altered expression in ovarian cancer of the remaining 20 genes is first reported here, although some, such as KLF4 [32], AKAP12 [38], NDRG2 [39, 40] and ADAMTS1 [41], have been linked to other types of human cancers. Kruppel-like factor 4 has been demonstrated as being mutated or down-regulated in several types of cancer including colorectal cancer, breast cancer, and bladder cancer [32, 42, 43]. Recent data implicate Kruppel-like factor 4 as a novel anti-inflammatory and anticoagulatory regulator of endothelial activation in response to pro-inflammatory stimuli [44]. A recent study indicates that AKAP12, a kinase-scaffolding protein, which is down-regulated in human breast, prostate, and gastric cancer, acts as a tumor and metastasis suppressor when re-expressed [38]. Other studies have shown that NDRG2, a cytosolic hydrolase, is expressed much higher in normal tissues than in tumors, where the expression is low or absent [39, 40]. This suggests that NDRG2 inactivation might play an important role in some tumor genesis or evolution, and that it acts as a cancer suppressor [45]. Our results also showed a significant down-regulatuion of ADAMTS1, a secreted-type of proteainase with thrombospondin motifs, which seems to be involved in tumor processes through its proteolytic activity and the regulation of cell adhesion [41]. Gene expression levels for five selected genes discussed here are represented in Figure 4. The classification ability and predictive power of these genes should be evaluated through further investigations.

The purpose of the competence net created in the course of this study was to enhance communication and collaboration between researchers, clinicians and patients. The network is specifically oriented towards expression analysis of ovarian serous carcinoma in the South Tyrolean population isolate, and brings together the most recent scientific findings and knowledge in this field. Together with our study centers in the regional hospitals we were able to validate the workflow of sample collection and sample selection for further studies. The possibility to standardise the diagnostic as well as the phenotypic criteria increases the reliability and the reproducibility of study results.

Our results provide a glimpse of the potential of gene expression profiling in population isolates for distinguishing epithelial ovarian cancers from normal ovarian epithelial cells. The elucidation of early molecular changes is urgently needed for the identification of prognostic factors and the development of suitable screening tools for the clinical management of ovarian cancer. One of the most challenging tasks when using microarrays is to determine which of the aberrantly regulated genes represent potential clinical utility. Through additional examination of the genes identified in this study it may be possible to choose a small number of candidate genes to be characterised in more detail. Although our data suggest the potential utility of this approach, we recognise that due to the limited sample size additional work is needed to fully take advantage of the distinctive research conditions found within our isolated population.

In conclusion, our statistical analysis highlights 40 genes with differential expression in ovarian serous papilloma cancer when compared to normal tissue. We have discussed the roles in the development of cancer of several genes which might provide further insights into the etiology of ovarian cancer and aid its clinical management.

Acknowledgments

The authors are grateful to the study participants and the hospitals of Bolzano, Merano, Brunico, and Bressanone (Departments of Gynaecology) for their participation and collaboration in this research project. We thank Prof. C. Wiedermann for helpful comments on the manuscript. The study was supported by the Ministry of Health of the Autonomous Province of Bolzano and the South Tyrolean Sparkasse Foundation.

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