Molecular and Cytogenetic Changes in Ovarian Carcinoma

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Academic Dissertation

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, referred to in the text by their Roman numerals.

I. Tapper J, Butzow R, Wahlström T, Seppälä M and Knuutila S: Evidence for divergence of DNA copy number changes in serous, mucinous and endometrioid ovarian carcinomas. Br J Cancer, 75: 1782-87, 1997.

II. Pere H*, Tapper J*, Seppälä M, Knuutila S and Butzow R: Genomic alterations in fallopian tube carcinoma; comparison to serous uterine and ovarian carcinomas reveals similarity suggesting likeness in molecular pathogenesis. Cancer Res, 58: 4274-4276, 1998.

III. Tapper J, Sarantaus L, Vahteristo P, Nevanlinna H, Hemmer S, Seppälä M, Knuutila S and Butzow R: Genetic changes in inherited and sporadic ovarian carcinomas by comparative genomic hybridization: Extensive similarity except for a difference at chromosome 2q24-q32. Cancer Res, 58: 2715-2719, 1998.

IV. Tapper J, Kettunen E, El Rifai W, Seppälä M, Andersson L. C and Knuutila S: Gene expression changes during progression of ovarian carcinoma. Cancer Genet Cytogenet, in press.

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ABBREVIATIONS

AIB1	amplified in breast cancer 1 gene
AKT2	gene encoding a serine-threonine protein kinase-related protein kinase c
BAC	bacterial artificial chromosome
BARD1	BRCA1-associated ring domain 1
BCL2	B-cell CLL/lymphoma-2 gene
BFL1	BCL2-related gene, murine A1 homolog
bp	base pair
BRCA1	breast cancer gene 1, early onset
BRCA2	breast cancer gene 2, early onset
cdc42	gene for cell division cycle 42 homolog
CDKN2	cyclin-dependent kinase inhibitor 2A gene, *MTS1*
cDNA	complementary DNA
CGH	comparative genomic hybridization
CK7	gene for keratin 7
CK18	gene for keratin 18
CK19	gene for keratin 19
CMET	hetocyte growth factor receptor gene
CMYC	v-myc avian myelocytomatosis viral oncogene homolog
C0L3A1	gene for collagen type 3 alpha 1
DAPI	4', 6-diamidino-2-phenylindole
DCC	deleted in colorectal cancer gene
dCTP	deoxycytidine triphosphate
DES	gene for desmin
DLG3	discs, large (Drosophila) homolog 3 gene, neuroendocrine-dlg
DNA	deoxyribonucleic acid
D0C2	differentially expressed in ovarian carcinoma gene
DOP	degenerate oligonucleotide-primed
dUTP	deoxyuridine triphosphate
ERBB2	avian erythroblastic leukemia viral oncogene homolog 2
EST	expressed sequence tag
FEZ1	gene for fasciculation and elongation protein zeta 1, zygin I
FGFRI	fibroblast growth factor receptor 1 gene
FIGO	International Federation of Gynecology and Obstetrics
FISH	fluorescence *in situ* hybridization
FITC	fluorescein isothiocyanate
FZD3	gene for frizzled, drosophila homolog 3
HGF	hepatocyte growth factor
HGFAC	HGF activator gene

HNPCC	hereditary nonpolypotic colorectal cancer
hRAD51	RAD51 (*S. cerevisiae*) homolog (*E. coli* RecA homolog), DNA repair protein rad51 homolog 1
HRAS1	Harvey rat sarcoma viral (v-Ha-ras) oncogene 1 homolog
HTLV-1	human T-cell leukemia virus 1
hTR	human telomerase RNA gene
IGFBP4	gene for insulin-like growth factor-binding protein 4
INT2	fibroblast growth factor 3 gene, murine mammary tumor virus integration site (v-int-2) oncogene homolog
kb	kilobase
KRAS	kirsten rat sarcoma 2 viral oncogene homolog
LMP	low malignant potential
LOH	loss of heterozygosity
MLH1	mutL (*E. coli*) homolog 1 gene
MMP2	matrix metalloproteinase 2 gene
MMP7	matrix metalloproteinase 7 gene
MSH2	mutS (*E. coli*) homolog 2 gene
MSH6	mutS (*E. coli*) homolog 6 gene
MSR	macrophage scavenger receptor gene
MTS1	cyclin-dependent kinase inhibitor 2A gene, *CDKN2*
N33	putative prostate cancer suppressor gene
0VCA1	ovarian cancer gene 1
0VCA2	ovarian cancer gene 2
p	short arm of the chromosome
PAC	P1 artificial chromosome
PCNA	gene for proliferative cellular nuclear antigen
PCR	polymerase chain reaction
PDGFRA	gene for platelet-derived growth factor receptor alpha
PIK3CA	phosphatidylinositol 3-kinase gene
PMS1	post-meiotic segregation increased (*S. cerevisae*)-like 1 gene
PMS2	post-meiotic segregation increased (*S. cerevisae*)-like 2 gene
q	long arm of the chromosome
RB1	retinoblastoma-1 gene
RhoGDI1	GDP dissociation inhibitor 1 gene
RNA	ribonucleic acid
Smad4	MAD (mothers against decapentaplegic, Drosophila) homolog 4 gene
TGF	transforming growth factor
TIMP2	tissue inhibitor of metalloproteinase 2 gene
TP53	gene for tumor protein p53
TRITC	tetramethylrhodamine
VEGF	vascular endothelial growth factor gene

INTRODUCTION

Ovarian cancer is the fourth most common cancer among women in Finland. It carries poor prognosis because the diagnosis is often delayed until there is advanced disease. Early detection is hampered by the lack of efficient diagnostic and screening methods, and the disease remains asymptomatic until advanced. Treatment comprises surgery and chemotherapy. Despite improvements, the overall prognosis has remained poor.

Ovarian carcinoma exhibits a wide spectrum of histological subtypes and biological behavior, ranging from aggressive and unresponsive to chemosensitive tumors. The most important prognostic indicators are clinical stage, histological subtype and grade, extent of residual tumor, patient's age and performance status (Friedlander, 1998). Even histopathologically identical tumors can differ in their clinical behavior. The natural course of the disease and its responsiveness to therapy can be unpredictable. Better understanding of the biological characteristics of ovarian carcinoma is needed to develop more individualized treatment modalities.

Despite rapid progress in cancer research, the genetic alterations in ovarian cancer are still incompletely understood. An important contribution was the identification of the tumor suppressor genes *BRCA1* and *BRCA2*. Inherited breast and ovarian cancers are known to be associated with mutations in these genes. Ovarian carcinoma can also be a manifestation of hereditary nonpolypotic colorectal cancer (HNPCC) syndrome. Many other cancer genes involved in ovarian cancer, however, still remain to be disclosed.

In the past few years, the development of new genome-wide screening techniques, such as comparative genomic hybridization (CGH), has played an important role in localizing possible cancer-associated genes. CGH has facilitated identification of chromosomal imbalances in human cancers (Kallioniemi et al., 1992; du Manoir et al., 1993). Another new approach is the so called microarray technique, which allows profiling of large scale gene expression.

This study is focused on molecular cytogenetic and molecular genetic screening and characterization of various types of ovarian carcinoma.

REVIEW OF THE LITERATURE

This thesis deals with epithelial ovarian cancer, and later in the text ovarian cancer and ovarian carcinoma refer to epithelial ovarian cancer.

1. Epidemiology of ovarian cancer

In Finland, 620 new cases of ovarian cancer were diagnosed in 1997 (The Finnish Cancer Registry, 2000). The age-standardized incidence in 1997 was 143/1,000,000 (adjusted for age to the World Standard Population) (The Finnish Cancer Registry, 2000). The cumulative incidence of ovarian cancer by the age of 75 years is 1.4% (Auranen et al., 1996).

2. Etiology of ovarian cancer

The causes of ovarian carcinoma are poorly understood. The "incessant ovulation" hypothesis of epithelial ovarian cancer etiology suggests that repeated rupture of the ovarian surface epithelium, followed by proliferation of the epithelial cells, could induce malignant transformation of these cells (Fathalla, 1971). According to the gonadotropin hypothesis, high gonadotropin levels increase the risk of cancer by stimulating the surface epithelium of the ovary (Stadel, 1975). Pituitary gonadotropins have been shown to stimulate the growth of human ovarian carcinoma cell lines *in vitro* (Simon et al., 1983). In addition, it is thought that the ovary can be exposed to external carcinogens ascending through the vagina and fallopian tubes.

3. Histology of ovarian cancer

Epithelial ovarian cancer accounts for about 90% of primary ovarian malignancies. It is thought to derive from the germinal surface epithelium of the ovary. During organogenesis the ovarian surface epithelium arises from the coelomic epithelium. Ovarian carcinoma is a heterogeneous group of tumors with several histological subtypes. The most common is ovarian serous carcinoma, which histologically resembles fallopian tube epithelium and represents approximately 40–53% of all ovarian carcinomas. The next most common are ovarian endometrioid carcinoma, which histologically resembles uterine endometrium (10–24% of cases), and ovarian mucinous carcinoma, resembling the uterine endocervix (3–14% of cases). Other histological subtypes consist of clear cell carcinoma (5–11%), malignant Brenner tumor (2%), malignant mixed epithelial tumor (5%) and undifferentiated carcinoma (6%) (Blaustein, 1982; Heinz et al., 2001).

4. Natural course and prognosis of ovarian cancer

Usually, ovarian carcinoma manifests after the menopause, the mean age at onset being 62 years (Dickman et al., 1999). Ovarian carcinoma can, however, occur in very young patients (Heinz et al., 2001), especially in the case of inherited ovarian cancer (Bewtra et al., 1992).

The overall prognosis of ovarian carcinoma is poor, the five-year survival rate be-

ing 48% (Heinz et al., 2001). Of the most common histologic subtypes, mucinous carcinoma has the best prognosis, the five-year survival rate being 69% (Heinz et al., 2001). The rates for serous and endometrioid carcinomas are 40% and 60%, respectively (Heinz et al., 2001). The survival rate improved slowly but steadily during the 1990s (Dickman et al., 1999; Heinz et al., 2001). The low survival rate reflects the aggressive nature of the disease and delay in diagnosis. Thus, about 70% of ovarian cancers are advanced at the time of diagnosis. The suitability of assay of tumor marker CA125 and abdominal or vaginal ultrasonography have been tested for screening of ovarian cancer, but due to the low incidence and often rapid natural course of the disease, they are not suitable for mass screening (Jacobs et al., 1999). In addition, assay of CA125 and ultrasonography are not sensitive enough for screening purposes.

5. Inherited ovarian cancer

In addition to sporadic forms of ovarian cancer, familial and inherited forms can be distinguished. In the familial form, usually several cancer cases occur in a family but the genetic background is unknown. In the inherited form, the inherited mutation in the predisposing gene, or other genetic factor, is known. It has been estimated that 5–10% of all ovarian carcinomas are associated with inherited germ-line mutations of cancer-predisposing genes (Boyd and Rubin, 1997). Here, two distinct phenotypes can be identified. The first is the breast and ovarian cancer syndrome, and the second is ovarian cancer associated with the hereditary nonpolypotic colorectal cancer (HNPCC) syndrome. The breast and ovarian cancer syndrome is linked to either the *BRCA1* gene at 17q21 (Miki et al., 1994) or the *BRCA2* gene at 13q12-q13 (Wooster et al., 1994). HNPCC tumors are linked to germ-line mutations in the mismatch repair genes *MLH1* (Papadopoulos et al., 1994), *MSH2* (Leach et al., 1993), *MSH6* (Edelmann et al., 1997; Miyaki et al., 1997), *PMS1* and *PMS2* (Nicolaides et al., 1994). The BRCA1 protein is a component of an RNA polymerase II transcription complex, and it is thought to function as a transcription factor (Scully et al., 1997). It has been suggested that BRCA1 could be related to cell differentiation and proliferation (Marquis et al., 1995). An additional suggested function of BRCA1 is in the maintenance of genomic stability, by repairing, together with hRad51 protein, double-strand DNA breaks (Scully et al., 1997). The results of loss of heterozygosity (LOH) analysis and functional studies suggest that *BRCA1* may act as a tumor suppressor gene (Boyd and Rubin, 1997). Like *BRCA1*, *BRCA2* is classified as a tumor suppressor gene (Boyd and Rubin, 1997). In addition, BRCA2 interacts with hRad51 protein and is involved in the repair of DNA breaks (Sharan et al., 1997). Thus, BRCA1 and BRCA2 proteins share similar functions.

The results of several studies suggest that the age at onset of inherited ovarian carcinoma is lower than that of sporadic ovarian carcinoma, and the clinical course of *BRCA1*-associated ovarian cancer is more favorable than that of sporadic cancer (Bewtra et al., 1992; Rubin et al., 1996). However, the prognosis of disease in *BRCA1* mutation carriers appears to be similar to, or even worse than that of sporadic ovarian cancer (Johannsson et al., 1998; Pharoah et al., 1999). It is of interest that most *BRCA1*- and *BRCA2*-associated ovarian carcinomas exhibit serous histology (Narod et al., 1994; Rubin et al., 1996), and among all forms of inherited ovarian cancer, mucinous carcinoma and borderline tumors are rare (Boyd and Rubin, 1997). Within a given histological subtype, inherited carcinoma is microscopically indistinguishable from sporadic carcinoma (Auranen et al., 1997).

6. Protective and risk factors of ovarian cancer

6.1. Sporadic ovarian cancer

Multiparity (Risch et al., 1983; Whittemore 1993; Adami et al., 1994), lactation (Whittemore 1993), oral contraceptive use (Risch et al., 1983; Whittemore 1993; Vessey and Painter, 1995), tubal ligation and hysterectomy (Kreiger et al., 1997; Miracle-McMahill et al., 1997) have been related to a decreased risk of ovarian cancer. Behavioral characteristics, such as use of cosmetic talc on the perineum is related to an increased risk (Whittemore et al., 1988; Harlow et al., 1992; Cramer et al., 1999). The risk factors associated with the different histological subtypes may vary (Kvale et al., 1988; Risch et al., 1996). There is evidence indicating that pregnancy and the use of oral contraceptives do not give protection against mucinous cancer (Risch et al., 1996).

6.2. Familial ovarian cancer

Of the factors other than age, a positive family history of ovarian cancer confers the greatest risk of ovarian cancer (Amos and Struewing, 1993; Parazzini et al., 1991; Schildkraut and Thompson, 1988). Women who have first degree relatives (mother, sister or daughter) with ovarian cancer have a two- to five-fold risk of ovarian cancer (Schildkraut and Thompson, 1988; Hartge et al., 1989; Parazzini et al., 1992; Auranen et al., 1996).

6.3. Inherited ovarian cancer

Epidemiological analysis suggests that there are families with a high incidence of breast cancer and a low incidence of ovarian cancer, and families with an equally high incidence of breast and ovarian cancer (Easton et al., 1995). Here, ovarian cancer risk may depend on the localization of the mutation in the *BRCA1* or *BRCA2* gene (Gayther et al., 1995; Gayther et al., 1997). By the age of 70 years, in *BRCA1* mutation carriers, the cumulative risk of ovarian cancer has been estimated to be 26–85% (Easton et al., 1993; Ford et al., 1994; Easton et al., 1995; Ford et al., 1995). In *BRCA2* mutation carriers, the risk of ovarian cancer is estimated to be less than 10% (Ford and Easton, 1995). The risk of ovarian cancer is two-fold in *BRCA1* mutation carriers who also have one or two rare alleles of the *HRAS1* gene at 11p15.5 (variable number of tandem repeats locus) (Phelan et al., 1996). In HNPCC kindreds, the estimated cumulative risk of ovarian cancer is 9% (Aarnio et al., 1995). Overall, the current risk estimates are based on studies of high risk families collected for research purposes and, thus, they may represent an overestimate of the cancer risk associated with *BRCA1* and *BRCA2* mutations.

7. Carcinogenesis

The genetic model of carcinogenesis has been thoroughly investigated in colorectal cancer (Fearon et al., 1990; Vogelstein and Kinzler, 1993). Here, the gradually accumulated genetic aberrations induce transformation of normal colonic epithelium, first to benign adenoma and eventually to carcinoma. In ovarian carcinoma, our knowledge of carcinogenesis is still limited. Only a minor subset of tumors with benign or low malignant potential are known to progress to invasive carcinoma (Chuaqui et al., 1997).

Carcinogenesis is a multi-step process. It has been estimated that, depending on the cancer type, 3–12 mutations are needed for carcinogenesis to take place (Renan, 1993). During cancer development genetic changes can accumulate in proto-oncogenes, tumor suppressor genes and DNA repair genes. Proto-oncogenes can be activated to oncogenes by structural changes, point mutations or amplifications, and lead to changes in gene expression or proteins.

Oncogenes are thought to have dominant action at the cellular level, since alterations in a single allele can cause malignant transformation in the cell. Gene amplifications can have clinical consequences, as shown in patients with progressing solid tumors and poor survival (Schwab and Amler, 1990). Tumor suppressor genes, also called "gatekeepers" (Kinzler and Vogelstein, 1997), suppress the development and progression of the malignant process. According to the two-hit theory, both alleles of a gene have to be altered to inactivate the gene (Knudson, 1971; Cavenee et al., 1983). Usually, only one allele is mutated, somatically or by an inherited trait, while the other allele is deleted by another event. The mechanism of allelic deletion may be loss of the whole chromosome or part of it, or mitotic recombination (Cavenee et al., 1983). The DNA repair genes, also called "caretakers" (Kinzler and Vogelstein, 1997), act in the recognition and repair of DNA damage. Germ-line mutations in the mismatch repair genes are linked to the HNPCC syndrome (Leach et al., 1993; Nicolaides et al., 1994; Papadopoulos et al., 1994; Edelmann et al., 1997; Miyaki et al., 1997).

8. Methods for genome-wide screeningof DNA changes

8.1. Chromosome analysis

In human malignancies, chromosomal imbalance in dividing cells has been widely studied using conventional chromosome analysis. Chromosomal examination of tumor requires culturing of tumor cells, which are then arrested in the metaphase or prometaphase and stained for analysis. The most common staining technique is the Giemsa method (G-banding). Cytogenetic analysis has proved to be valuable in studying hematological malignancies. Much fewer cytogenetic data are available on solid tumors, especially carcinomas. This is due in part to technical difficulties in studying neoplastic epithelial cells, and to problems in obtaining good quality metaphase spreads for precise identification of chromosomal changes. Tumor karyotypes are often very complex, containing many numerical and structural abnormalities that make interpretation obscure. In addition, those cells that can be cultured may not necessarily represent the major tumor cell clone. Some chromosomal changes, such as small deletions and gene amplifications may remain undetected. Moreover, identification of the genomic origin of the double minute chromosomes and homogeneously staining regions, the cytogenetic manifestations of gene amplification, cannot be carried out by banding analysis. (Heim and Mitelman, 1995).

8.2. Fluorescence in situ hybridization (FISH)

Fuorescence *in situ* hybridization (FISH) can be used to complement conventional chromosome banding analysis. It allows higher resolution of the chromosomal changes. In addition, FISH can be used for studying structural changes, such as translocations, which may be difficult to find by conventional cytogenetic analysis. Several applications of the FISH technique have been developed. In chromosome painting, the DNA probe is composed of a collection of different DNA fragments from an individual chromosome and, thus, the whole chromosome will be stained. Chromosome painting is useful for defining marker chromosomes and recognizing chromosome segments. Furthermore, instead of using one or two colors for detection of one or two targets, several targets can be detected by combining different fluorescent dyes (Ried et al., 1992). New modifications of this technique are multicolor FISH (Speicher et al., 1996) and spectral karyotyping (Schrock

et al., 1996), in which different chromosomal regions can be stained in various colors. These techniques are useful for characterization of marker chromosomes and detection of subtle chromosomal aberrations.

8.3. Comparative genomic hybridization (CGH)

Comparative genomic hybridization was introduced in 1992 by Kallioniemi and his co-workers. The method allows whole genome detection and mapping of DNA sequence copy number changes in a single experimental setting. It is based on simultaneous hybridization of differentially fluorescently labeled test and normal DNAs to normal metaphase chromosomes (Figure 1). The differences in binding of the test versus normal DNA sequences to normal metaphase chromosomes reflect the DNA copy number variation between the test and the reference genomes. The fluorescence intensity ratios are measured in a digital image analysis system. An increased test to reference fluorescence intensity ratio suggests gains or amplifications of DNA copy number sequences in the test sample, whereas a decreased ratio indicates losses of the same (Figure 1).

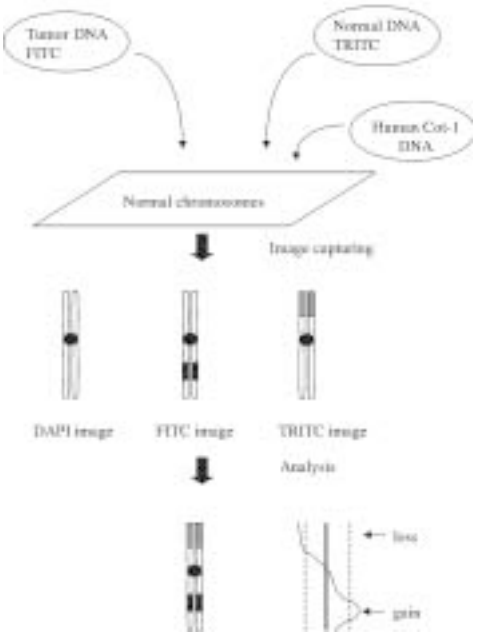

Figure 1. The principle of comparative genomic hybridization. Differentially labeled tumor and normal DNAs are hybridized with Human Cot-1 DNA in normal metaphase chromosomes. Three digital images are captured; one DAPI counterstain image, one FITC image for tumor DNA and one TRITC image for normal DNA. Differences in the tumor to normal fluorescence ratio on the chromosomes reflect DNA copy number changes in the tumor sample. The ratio difference is shown as a profile.

The CGH method facilitates the study of tumor samples for changes in DNA sequence copy number, whereas balanced translocations, inversions or ploidy changes are not detectable. The technique allows detection of the changes present in a substantial proportion of tumor cells, provided that the tumor cells predominate. The sensitivity of the method depends on the size and the magnitude of the copy number aberration (Kallioniemi et al., 1994). Deletions of 10–20 Mb in size have been reliably detected by CGH (Bentz et al., 1998). On the other hand, if the sequence is highly amplified (up to 5–10 fold), changes as small as 1 Mb in size can be seen (Forozan et al., 1997). Some genomic areas, such as the pericentromeric and the heterochromatic regions cannot be evaluated reliably because they contain highly repetitive sequences which are blocked by unlabeled human Cot-1 DNA. In telomeric regions, the ratio changes should be interpreted with caution because the fluorescence intensities gradually decrease towards the telomeres, approaching the background and, thus, unreliable results may be obtained (Kallioniemi et al., 1994).

Several improvements have been made in this methodology. Direct fluorochrome-conjugated nucleotides have replaced the indirect labeling system, improving reso-

lution and sensitivity (Kallioniemi et al., 1994). The use of paraffin-embedded tumor material has allowed retrospective analysis of archival tumors (Speicher et al., 1993; Isola et al., 1994). Degenerate oligonucleotide-primed PCR has facilitated the use of very small amounts of DNA for analysis (Speicher et al., 1993; Kuukasjärvi et al., 1997). Recently, CGH has been successfully used to study DNA from one cell only (Klein et al., 1999). Ratio artefacts, which may occur in the GC- rich genomic regions, can be minimized by using a mixture of dCTP and dUTP nucleotides in the labeling of probes (El Rifai et al., 1997). Four-color CGH modification was developed to identify inconsistently hybridized chromosomal regions and to standardize the hybridization dynamics, using internal control DNA (Karhu et al., 1999). The resolution of CGH has been too low to detect small amplifications or to distinguish high-level amplification from a low-level gain of a larger segment (Tanner et al., 2000). To overcome the limited resolution of chromosomal CGH, array-based CGH has provided a means to increase the mapping resolution (Solinas-Toldo et al., 1997; Pinkel et al., 1998; Pollack et al., 1999). In array-based CGH, cosmids, PAC and BAC clones and cDNAs can be used as targets of 0.5–2 kb in size (Pinkel et al., 1998; Pollack et al., 1999).

8.4. Southern blot hybridization

Southern blot hybridization using gene- or sequence-specific probes is suitable for studying gene amplifications, deletions and rearrangements. In Southern blot hybridization, size-fractionated, denaturated target DNA is transferred by blotting from agarose gel to a nylon or nitrocellulose membrane. Subsequently, the immobilized, single stranded target DNA is hybridized using radioactively labeled probes. The probe will bind to complementary DNA sequences in the target DNA only. The intensity of the hybridization signal is quantified by autoradiography and densitometric scanning, or by phosphor imaging.

8.5. Loss of heterozygosity (LOH)

Loss of heterozygosity (LOH) analysis is used for identifying allelic losses. In the analysis, the polymerase chain reaction (PCR) is used to detect the highly polymorphic microsatellite markers in paired normal and tumor tissues. The analysis is based on comparison of marker alleles in paired normal and tumor DNA samples. If the normal DNA is heterozygous for a given marker and the tumor sample is homozygous, it is considered as a loss of heterozygosity. This can pinpoint the region possibly harboring a tumor suppressor gene.

8.6. A new genome screening method, using a cDNA microarray technique

Like the chromosomal CGH technique, developed to an array, molecular analyses have been similarly carried out by using different arrays to obtain large scale information on gene expression or protein localization (Schena et al., 1995; DeRisi et al., 1996; Schena et al., 1996; Kononen et al., 1998). A new approach is the so-called complementary DNA (cDNA) microarray technique, which allows simultaneous expression analysis of hundreds or even thousands of genes in the sample of interest (Schena et al., 1995; DeRisi et al., 1996; Schena et al., 1996). In the cDNA array, cDNA sequences can be immobilized on a glass slide or a nylon membrane, and hybridized with a labeled probe produced from RNA of a tissue, cell line, or organism of interest, to create a gene expression fingerprint (Figure 2). Amplification of RNA has enabled study of even small cell populations microdissected from a tissue section (Gonzalez et al., 1999; Luo et al., 1999).

This large scale expression survey has

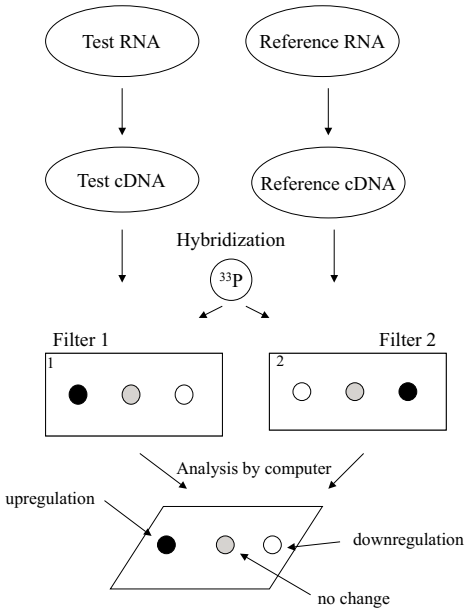

Figure 2. The principle of the cDNA array technique. RNAs from test and reference samples are converted to cDNA. The cDNAs are labeled with radioactive ^{33}P . The probes are hybridized to identical nylon membranes (arrays) containing known cancer-related genes. After hybridization, the membranes are exposed to an imaging plate and subsequently scanned for computer analysis. In the analysis, the membranes are overlapped for comparison

been applied to investigate gene expression changes in different human cancers. Recently, studies have been performed on several cell lines, such as alveolar rhabdomyosarcoma cell line (Khan et al., 1998), HTLV-1-immortalized T cells (Harhaj et al., 1999), a glioblastoma cell line (Rhee et al., 1999), and an ovarian cancer cell line (Ismail et al., 2000). Gene expression profiles of 16 primary ovarian carcinomas have been reported (Wang et al., 1999; Ono et al., 2000).

9. Chromosomal imbalance in ovarian carcinoma

Cytogenetic studies of ovarian carcinoma have revealed highly complex karyotypes with multiple numerical and structural alterations, and with double minute chromosomes and homogeneously staining regions. Cytogenetically abnormal cases have been detected in 50–80% of cases (Pejovic et al., 1992; Jenkins et al., 1993; Taetle et al., 1999a). The majority have displayed complex karyotypes; only a few tumors exhibit simple changes, e.g. numerical changes only, or a single structural change, or both. In simple cases, the most common change is to trisomy of chromosome 12 (Pejovic et al., 1992a; Jenkins et al., 1993). In some benign, borderline and malignant tumors, trisomy 12 is the only cytogenetic aberration present (Pejovic et al., 1990; Pejovic et al., 1992a; Jenkins et al., 1993; Thompson et al., 1994). Frequently detected aberrations have been detected at chromosomes 1, 3, 6, 7, 11, 12, 19 and X (Wake et al., 1980; Tanaka et al., 1989; Roberts and Tattersall, 1990; Pejovic et al., 1992a; Jenkins et al., 1993; Thompson et al., 1994; Taetle et al., 1999a). Aberrations of chromosome band 19p13 are relatively the most common (Pejovic et al., 1992a; Jenkins et al., 1993; Thompson et al., 1994). The findings, however, have varied in different studies, probably reflecting limited numbers of observations, complexity of changes, or methodological difficulties. Double minutes and homogeneously staining regions have been reported with variable frequencies (Tanaka et al., 1989; Bello and Rey, 1990; McGill et al., 1993; Thompson et al., 1994). The clinical significance of cytogenetic changes has been assessed in some studies (Roberts and Tattersall, 1990; Pejovic et al., 1992b; Taetle et al., 1999b). In the largest panel of samples (n=244), breakpoints at 1p1 and 3p1 were associated with poor survival (Taetle et al., 1999b).

Cytogenetic abnormalities are more common in seropapillary tumors and in cases at an advanced stage and with residual disease after primary surgery (Pejovic et al., 1992b). Complex changes correlate with poor histological differentiation and/or unfavorable outcome (Pejovic et al., 1992b; Taetle et al., 1999b). Overall, complex karyotypes are characteristic of tumors with moderate or poor histological differentiation (Pejovic et al., 1992a; Pejovic et al., 1992b; Thompson et al., 1994).

10. Molecular genetic changes in ovarian carcinoma

10.1. Sporadic ovarian carcinoma

As in other cancers, the roles of both oncogenes and tumor suppressor genes have been studied in ovarian carcinoma. The *ERBB2* gene is located at 17q12 and encodes a cell surface protein homologous to epidermal growth factor receptor. Overexpression or gene amplification of *ERBB2* has been reported in 18–32% of ovarian carcinomas (Slamon et al., 1989; Berchuck et al., 1990; Zheng et al., 1991; Rubin et al., 1993; Young et al., 1996). It has been associated with poor prognosis (Slamon et al., 1989; Berchuck et al., 1990), but later studies have not confirmed the clinical impact of overexpression of ERBB2 (Rubin et al., 1993; Singleton et al., 1994).

The *KRAS* gene is a member of the *ras* gene family and is located at 12p12.1. It encodes p21/k-ras 2a transforming protein, localized to the cytoplasmic side of the inner plasma membrane, and appears to be involved in cell surface receptor signal transduction. In epithelial ovarian tumors, mutations of the *KRAS* gene are more common in mucinous tumors than in other histological subtypes (Enomoto et al., 1990; Ichikawa et al., 1994). *KRAS* mutations have also been detected in benign adenomas and borderline tumors with mucinous histology (Mok et al., 1993; Teneriello et al., 1993; Ichikawa et al., 1994; Chenevix-Trench et al., 1997).

Several other oncogenes have been shown to be amplified and/or overexpressed in ovarian carcinoma, such as *AIB1* (in 25%) (Tanner et al., 2000), *CMYC* (25–38%) (Zhou et al., 1988; Baker et al., 1990; Sasano et al., 1990; Tashiro et al., 1992), *INT2* (19%) (Medl et al., 1995), *AKT2* (12–13%) (Bellacosa et al., 1995; Cheng et al., 1992) and *CMET* (28%) (Di-Renzo et al., 1994).

Some tumor suppressor genes have been found to be involved in ovarian carcinoma. Mutations of the tumor suppressor gene *TP53* at 17p13.1 are probably among the most common genetic alterations found in human cancer. In ovarian carcinoma, the mutant TP53 protein has been detected in about half of the tumors (Marks et al., 1991; Hartmann et al., 1994; Klemi et al., 1995). Mutations and overexpression have been detected in advanced stage cancer more frequently than in early stage cancer. Overexpression of TP53 is associated with serous histology, unfavorable outcome and poor histological differentiation (Kupryjanczyk et al., 1994; Klemi et al., 1995). In benign cystadenoma, no overexpression has been found (Marks et al., 1991). Positive immunostaining, without mutations, has been detected in borderline tumors (Kupryjanczyk et al., 1994; Kupryjanczyk et al., 1995).

The roles of other tumor suppressor genes, such as *RB1* (13q14), *CDKN2* (9p21), *D0C2* (5p13), *BRCA1* and *BRCA2* have also been studied. The role of *RB1* seems to be insignificant, but inactivation of *CDKN2* occurs in about 20% of ovarian tumors, especially in endometrioid and mucinous tumors (Sasano et al., 1990; Milde-Langosch et al., 1998). The *D0C2* gene has been suggested to be involved in serous ovarian carcinoma (Mok et al., 1998). Mutations in *BRCA1* and *BRCA2* genes are rare in sporadic ovarian carcinoma (Futreal et al., 1994; Merajver et al., 1995; Takahashi et al., 1995; Foster et al., 1996; Lancaster

Table 1. CGH studies on ovarian carcinoma. The number and frequency of abnormal cases.

Reference	Number of cases studied	Number of abnormal cases in CGH (%)
Arnold et al., 1996	47	45 (96%)
Blegen et al., 2000	24	not available
Iwabuchi et al., 1995	44	not available
Kiechle et al., 2001	106	103 (97%)
Kudoh et al., 1999	28	28 (100%)
Pejovic et al., 1999	2	2 (100%)
Sonoda et al., 1997	25	22 (88%)
Suzuki et al., 2000	40	not available
Wolff et al., 1997	24	24 (100%)

et al., 1996; Takahashi et al., 1996).

10.2. Inherited ovarian carcinoma

Some somatic molecular genetic changes have been studied in *BRCA1* and *BRCA2* mutation-associated inherited ovarian carcinomas. *TP53* mutations have been found in 80% of the cases (83% of *BRCA1* tumors; 73–78% of *BRCA2* tumors) (Rhei et al., 1998; Ramus et al., 1999). *TP53* mutations appear to be more common in *BRCA1*- and *BRCA2*-associated ovarian carcinoma than in sporadic tumors (Ramus et al., 1999). Point mutations of *KRAS* at codon 12, or amplification of *ERBB2*, *CMYC* or *AKT2*, which are common in sporadic ovarian carcinoma, have not been found (Rhei et al., 1998; Tanner et al., 2000).

11. DNA sequence copy number changes in ovarian tumors

Iwabuchi and co-workers (1995) published the first CGH study on ovarian carcinoma. Before this study was launched, reports on genome-wide screening of DNA sequence gains and losses in ovarian carcinoma were not available. So far, some 340 cases of ovarian carcinoma have been studied using CGH (Iwabuchi et al., 1995; Arnold et al., 1996; Sonoda et al., 1997; Wolff et al., 1997; Kudoh et al., 1999; Pejovic et al., 1999; Blegen et al., 2000; Suzuki et al., 2000; Kiechle et al., 2001) (Table 1). Chromosomal changes have been found in 85–100% of cases, which is slightly more than in standard cytogenetic studies (Table 1). This is probably due to the improved technology achieved using CGH compared with cytogenetic methods. The findings in CGH studies are referred to later in the Discussion.

In addition to ovarian carcinoma, CGH analysis has been performed on benign ovarian tumors. Results have been published on 37 ovarian adenomas and tumors with low malignant potential (LMP) (Iwabuchi et al., 1995; Arnold et al., 1996; Sonoda et al., 1997; Wolf et al., 1999; Blegen et al., 2000). Many of the tumors have shown alterations consistent with those in carcinoma, suggesting the possibility that at least some of the adenomas and LMP tumors may be precursors of carcinoma. It is possible that there are genomic changes in other benign tumors as well, but CGH analysis is easily

Table 2. The most common chromosomal gains and losses in 299 ovarian carcinomas detected by using CGH (Arnold et al., 1996; Blegen et al., 2000; Iwabuchi et al. 1995; Kiechle et al., 2001; Kudoh et al., 1999; Pejovic et al., 1999; Sonoda et al., 1997; Wolff et al., 1997).

Gains	Frequency	Losses	Frequency
+8q22-qter	58%	-4q22-qter	32%
+3q24-qter	51%	-13q	32%
+1q21-qter	46%	-18q12-qter	31%
+20q11.2-qter	42%	-8p21-pter	28%
+12p	32%	-16q	24%
+1p13-p35	29%	-17p	20%
+7q21-qter	29%	-17q	19%
+6p12-pter	28%	-Xp	16%
+5p	28%	-Xq	14%

compromised by contamination with normal cells. Twenty-one of the 37 tumors were microdissected to enrich the tumor cell proportion (Wolf et al., 1999; Blegen et al., 2000).

12 . LOH in ovarian carcinoma

Numerous LOH studies on ovarian carcinoma have been carried out to pinpoint the regions possibly harboring tumor suppressor genes. Studies have been performed either by 1) mapping a chromosome with several markers to narrow down the critical region, or 2) screening with only a few markers per chromosome to survey several chromosomes for losses. In ovarian carcinoma, varying frequencies of allelic loss have been observed in all chromosomes (Sato et al., 1991; Cliby et al., 1993; Dodson et al., 1993; Yang-Feng et al., 1993; Launonen et al., 2000). LOH has been found at several genomic loci. Frequently lost regions have been chromosomal arms 3p, 6p, 6q, 11p, 13q, 14q, 17p, 17q, 22q and Xp (Sato et al., 1991; Eccles et al., 1992; Yang-Feng et al., 1992; Cliby et al., 1993; Dodson et al., 1993; Foulkes et al., 1993a; Foulkes et al., 1993b; Weitzel et al., 1994; Phillips et al., 1996; Bandera et al., 1997; Fullwood et al., 1999; Bryan et al., 2000; Launonen et al., 2000; Lin et al., 2000). However, LOH at chromosome 17 has consistently been the most common finding.

Chromosomal arm 17p has been screened for LOH using several markers, and there is evidence for the presence of other tumor suppressor genes, apart from *TP53*. Schultz and co-workers have defined the minimum region of allelic loss at 17p13.3 between markers D17S5 and D17S28, the estimated genetic distance being 3.5 cM. In Northern analysis, mRNA expression of candidate genes *OVCA1* and *OVCA2*, located within the above-mentioned region, is decreased in ovarian carcinoma (Schultz et al., 1996).

The pattern of LOH correlates with the histopathological characteristics of the tu-

mor. Thus, LOH is more common in serous than in nonserous tumors, and it is more common in poorly differentiated than in highly differentiated tumors (Sato et al., 1991; Cliby et al., 1993; Dodson et al., 1993; Saretzki et al., 1997). Deletions at chromosome arms 6q, 13q, 17p and q, and 19q have been found more frequently in serous than in nonserous tumors (Sato et al., 1991; Saito et al., 1992; Pieretti et al., 1995). LOH at chromosome arm 3p has been detected more frequently in disseminated ovarian carcinomas than in local carcinomas (Fullwood et al., 1999). Furthermore, LOH at genomic regions 3p, 11p, 11q, 16q and 17p have been related to an adverse disease course in ovarian cancer patients (Launonen et al., 2000).

On the basis of LOH data, it is possible that inactivation of several tumor suppressor genes occurs during progression of ovarian carcinoma. Despite the fact that LOH is common in many genomic regions in ovarian carcinoma, the tumor suppressor genes in most regions have remained elusive.

In ovarian carcinoma, the concordance between CGH and LOH findings ranges from 56% to 100% (overall concordance 84%) depending on the locus (Iwabuchi et al., 1995). Only 31% of LOH has been associated with losses detected by CGH (Iwabuchi et al., 1995). However, the most frequently lost regions in CGH also exhibit LOH (Table 2). The discrepancy in certain loci partly reflects the differences in resolution between the methods, and on the other hand, the different mechanisms of LOH. If the LOH is due to physical deletion, CGH can reveal it. If the deletion is small or discontinous, or if the LOH is due to mitotic recombination, then CGH cannot detect it. If the LOH is due to loss of one allele and the other allele is amplified, then CGH may even indicate amplification.

AIMS OF THE STUDY

- to characterize the chromosomal changes in sporadic and inherited ovarian carcinomas, as well as in fallopian tube carcinoma (I-III)

- to compare chromosomal changes in different histological subtypes of ovarian carcinoma (I)

- to compare chromosomal changes in serous carcinoma of the ovary, fallopian tube and endometrium (II)

- to compare chromosomal changes in sporadic and inherited ovarian carcinoma (III)

- to screen and compare the gene expression changes in serous ovarian carcinoma of different stages and histological grades (IV)

MATERIALS AND METHODS

1. Patients and tissues (I-IV)

All the studies were approved by the Institutional Review Board of the Department of Obstetrics and Gynecology, Helsinki University Central Hospital. In study I, comparative genomic hybridization analysis was performed on 24 samples of ovarian carcinoma, including 8 serous, 8 endometrioid and 8 mucinous carcinomas of various stages, the patients being diagnosed and treated at the Department of Obstetrics and Gynecology, Helsinki University Central Hospital.

The material of study II consisted of 20 primary fallopian tube carcinomas, 20 ovarian carcinomas and 24 endometrial carcinomas (Pere et al., 1998), the patients being treated at the Department of Obstetrics and Gynecology, Helsinki University Central Hospital. All the tumors were of serous histology.

Study III comprised 20 inherited and 20 sporadic ovarian cancers. The samples from patients with inherited ovarian cancer were obtained from different hospitals in Finland. The patients were members of breast-ovarian cancer families previously analyzed for *BRCA1* and *BRCA2* mutations (Vehmanen et al., 1997a; Vehmanen et al., 1997b), or unselected mutation-positive ovarian cancer cases treated at the Department of Obstetrics and Gynecology, Helsinki University Central Hospital. Sixteen of these patients were *BRCA1* mutation-positive and four were *BRCA2* mutation-positive. Histologically, 14 tumors of the BRCA1 group were serous cystadenocarcinomas, one was an endometrioid adenocarcinoma and one was a borderline mucinous cystadenoma. In the BRCA2 group, three tumors were serous cystadenocarcinomas, one of them possibly originating from the fallopian tube, and one was an endometrioid adenocarcinoma. The "sporadic" group (tissues also used in study II) consisted of 20 serous ovarian cystadenocarcinomas of similar stage and grade. All the patients with sporadic cancer were treated at the Department of Obstetrics and Gynecology, Helsinki University Central Hospital.

The study IV material comprised 6 ovarian serous cystadenocarcinomas and 1 benign ovarian serous cystadenoma.

2. Methods

2.1. Comparative genomic hybridization (I-III)

Genomic DNA from frozen tissues and peripheral blood from healthy female donors was extracted by using standard methods. DNA from paraffin-embedded tissues was extracted according to the method described by Isola and co-workers (1994). Target metaphase slides were prepared from phytohemagglutin-stimulated blood lymphocytes from normal donors (46,XX or 46,XY).

Comparative genomic hybridization was performed as described previously (Kallioniemi et al., 1992; Kallioniemi et al., 1994), with slight modifications (Figure 1).

In study I, tumor DNA and normal DNA were labeled, by nick translation, with biotin-14dATP (Gibco BRL, Gaithersburg, MD) and digoxigenin-11dUTP

(Boehringer Mannheim, Mannheim, Germany), respectively. The amounts of DNAase and DNA polymerase I were adjusted to obtain probe fragments ranging from 600–2000 base pairs. Equal amounts (400 ng) of labeled tumor and normal female DNA, as well as 10 µg of unlabeled human Cot-1 DNA (Gibco BRL, Gaithersburg, MD), were hybridized to normal metaphase spreads at 37°C for 2–3 days. After hybridization, the preparations were washed to remove unbound DNA; three times in 50% formamide/2xSSC (pH 7.0), twice in 2xSSC, and once in 0.1xSSC, at 45°C for 10 minutes each. Tumor DNA was visualized with tetra-rhodamine isothiocyanate (TRITC) -conjugated avidin and normal DNA was detected with fluorescein isothiocyanate (FITC) anti-digoxigenin. The slides were counterstained with 4,6-diamidino-2-phenylindole (DAPI) and covered with antifade solution (Vectashield™, Vector Laboratories, Burlingame, CA) for identification of the chromosomes.

In studies II and III, the protocol was changed to the use of directly fluorochrome-conjugated nucleotides, to improve resolution and sensitivity (El Rifai et al., 1997; Kallioniemi et al., 1994). Briefly, 1 µg tumor DNA was labeled with FITC-12dUTP (Du Pont, Boston, MA) or with FITC-12dUTP and FITC-12dCTP (Du Pont) (1:1), and 1 µg normal DNA was labeled with Texas Red-5dUTP (Du Pont) or with Texas Red-5dUTP and Texas Red-5dCTP (Du Pont) (1:1) in standard nick translation. Equal amounts of labeled test and reference DNA were hybridized to normal metaphase spreads for 2–3 days. After hybridization, washing was carried out as described above, followed by washing in 2xSSC in PN buffer (mixture of 0.1 M NaH_2PO_4 and 0.1 M Na_2HPO_4, pH 8.0, and Nonidet P-40), and in distilled water, at room temperature for 10 minutes each.

In study III, the amount of DNA in one sample was insufficient for CGH. This sample was subjected to degenerate oligonucleotide primed-PCR (DOP-PCR), following the protocol described by Kuukasjärvi and her co-workers (1997), with some modifications. Briefly, 5 µl of DNA solution was used as a template for DOP-PCR with a universal primer 5'-CCGACTCGAGNNNNNNATGTGG-3' (N=A, C, G or T). Thermosequenase (Amersham, Cleveland, OH) in a buffer (10 mM Tris-HCl, pH 8.0, 1 mM 2-mercaptoethanol, 0.5% Tween-20, 0.5% Nonidet P40) was applied at 1:10 dilution (3 units/reaction) in a solution of 26 mM Tris-HCl, pH 9.5, 6.5 mM $MgCl_2$, 0.2 mM dNTPs, 1 µM universal primer, the total volume being 10 µl. The sample was subjected to six cycles of 94°C for 1 minute 30 °C for 3 minutes and 65°C for 5 minutes, with final extension at 72°C for 10 minutes followed by high stringency cycles consisting of initial melting at 95°C for 3 minutes, then 30–35 cycles of 94°C for 1 minute, 56°C for 1 minute and 72°C for 2 minutes, with final extension at 72°C for 5 minutes (Thermocycler PTC-200, MJ Research Inc. Watertown, MA), in a volume of 50 µl. The product was labeled with fluorescein 12-dUTP (Fluorescein-High prime kit, Boehringer Mannheim, Detroit, MI), using the standard priming method recommended by the supplier.

2.2. Digital image analysis in CGH (I-III)

The hybridizations were evaluated using a Leitz (study I) or Olympus (studies II, III) fluorescence microscope and an *ISIS* digital image analysis system (MetaSystems GmbH, Altlussheim, Germany), integrating a monochrome charge-coupled device camera and an automated CGH analysis software package. Three-color images, red (TRITC) for tumor DNA hybridization (study I), green (FITC) for normal reference DNA hybridization (study I) or for tumor DNA (FITC) (studies II and III), red (TRITC) for reference DNA (studies II and III) and blue (DAPI) for DNA counterstain,

were acquired from 5 to 10 metaphase spreads per hybridization (Figure 1). The fluorescent background was reduced by automatic background correction. The homogeneous background allowed chromosome segmentation by thresholding of the DAPI image. The chromosomes were identified and karyotyped on the basis of their DAPI-banding patterns. Red and green fluorescence intensities were measured and the red-to-green ratio (study I) or green-to-red ratio (study II) profiles along the medial axis from the p- to the q-telomere were displayed. For normalization of ratio profiles, the modal value of the red-to-green (study I) or green-to-red (studies II and III) ratio for the entire metaphase spread was set to 1.0. This was repeated to analyze the profiles of all the metaphases included in the analysis. The individual ratio profiles were combined using separate normalizations of the p- and q-telomeres to yield the average ratio profiles that were displayed simultaneously next to the ideograms, with significance intervals of 0.85 and 1.17, respectively. In studies II and III, all the findings were confirmed using a confidence interval of 99%.

The chromosomal regions with a red-to green ratio (study I) or a green-to-red ratio (studies II and III) exceeding 1.17 were considered to be over-represented (gains), whereas the regions with a ratio below 0.85 were considered to be under-represented (losses). These values were set on the basis of the results of negative control experiments where two differently labeled normal DNAs were hybridized together. In the negative controls, the ratios varied within these limits. Tumor DNA with a known number of copy alterations was used in positive control experiments. In study II, reverse labeling CGH was performed in selected cases, which confirmed the alterations detected by the standard technique. The cut-off level for high-level amplification was 1.5. Telomeric and heterochromatic areas were discarded from the analysis.

2.3. cDNA array (IV)

Frozen tissue samples were disrupted and homogenized using an Ultra Turrax (Ika Werk, Janke & Kunkel, Germany). Total RNA from the tissue samples was isolated by using Qiagen RNeasy Maxi Kit (Qiagen Inc., Santa Clarita, CA) following the manufacturer's instructions. After isolation, total RNA was purified from DNA by using DNaseI (Boehringer Mannheim, Mannheim, Germany) enzyme incubation, subsequently treating with phenol-chloroform-isoamylalcohol, precipitating, and resuspending in RNase-free H_2O. The integrity of the RNA was confirmed by electrophoresis on 1% agarose gel.

A sample of total RNA (approximately 4 µg) was incubated with CDS Primer (Clontech) at 70°C for 2 min and at 48°C for 2 min and subsequently reverse transcribed and labeled with ^{33}P dATP. The reverse-transcribed probe was purified using a Chroma Spin-200 column (Clontech), denaturated and neutralized. The radiolabeled cDNA probe was hybridized to a membrane containing known cancer-related genes (Atlas™ Human Cancer cDNA Expression Array, Clontech, Inc., CA), overnight at 68°C in a hybridization oven (Mini hybridization oven, Hybaid Inc.) with continuous rolling. After hybridization, the membrane was washed at 68°C in 2xSSC, 1% SDS for 2 hours, and in 0.1xSSC, 0.5% SDS for 1 hour. After the washes, the membrane was sealed in a plastic wrap to prevent drying, and exposed to a phosphor imaging plate (Fuji Film, Japan) for four to six days and scanned in a Bio-Imaging Analyzer (BAS-2500; Fuji, Nakanuma, Japan).

The membranes were reused 3 to 4 times. After exposure, they were stripped to remove the cDNA probe by boiling in 0.5% SDS solution for 10–15 minutes. The efficiency of stripping was checked by exposure to a phosphor imaging plate overnight.

A single membrane contained cDNA fragments from 588 human genes. Each

cDNA fragment contained 10 ng cDNA, and was immobilized in duplicate to ensure the quality of hybridization. The genes in the membrane were subdivided into six groups according to their function: a) cell cycle and growth regulators, and intermediate filament markers, b) apoptosis-related genes, oncogenes and tumor suppressor genes, c) DNA damage response genes, repair and recombination genes, cell fate and development genes, and receptors, d) cell adhesion and motility genes, e) invasion regulators and cell-cell interaction genes, and f) growth factors and cytokines. The list of genes is available at Clontech's web site (http://www.clontech.com).

2.4. cDNA array analysis (IV)

The hybridization signals were analyzed by using Atlas Image™ 1.0 software (Clontech). The two images to be compared were normalized for the calculations by selecting and utilizing 7 out of 9 housekeeping genes included in the membrane. In addition, the membrane contained three negative control DNAs in triplicate. The negative control spots had to be free of radioactive signals. The results were visually confirmed. Selective analyses were repeated to validate reliability and reproducibility of the method.

To create gene expression fingerprint profiles for ovarian carcinoma, the expression levels of the 588 known genes immobilized on the membrane were analyzed. The following comparisons were made: 1) ovarian adenocarcinoma versus benign adenoma, 2) local, highly differentiated adenocarcinoma versus benign adenoma, and 3) advanced and/or moderately or poorly differentiated ovarian adenocarcinoma versus local, highly differentiated ovarian tumor. The expression databases obtained from the comparisons were sorted the intensity ratio values, in descending order. The genes which showed an adjusted intensity ratio above 2 or less than 0.5 in four or more samples were considered to be differentially expressed.

2.5. RT-PCR (IV)

RT-PCR was used to confirm the cDNA array results of two genes, *C0L3A1* and *RH0GDI2*. For reverse transcription reactions we used random hexamer primers and SuperScript™ II RNase H$^-$ Reverse Transcriptase (Gibco BRL, MD, USA). Gene-specific PCR primer sequence information was acquired from Clontech. As a reference we used glyceraldehyde 3-phosphate dehydrogenase (*G3PDH*). The 50 µl PCR reaction mixtures contained 1.0 unit of Perkin Elmer AmpliTaq Gold™ (Roche Molecular Systems, Inc., NJ, USA) in 1x buffer (GeneAmp 10x PCR Buffer II, Roche), and 1.5 mM magnesium chloride (Roche). The reaction contained dNTPs (each 0.25 mM) (Finnzymes, Espoo, Finland) and 20 pmol of each primer. The PCR reactions included initial denaturation at 95°C for 10 min, followed by 25 to 30 cycles of 1 min at 95°C, 1 min at 56°C, and 1 min at 72°C, with 10 min final extension at 72°C.

2.6. Statistical analysis (III)

The differences in the frequency of copy number changes between BRCA1 and "sporadic" groups were tested by using Fisher's exact test with two-tailed p-values.

RESULTS

1. Summary of CGH results (I-III)

Altogether, 61 ovarian carcinomas were studied by CGH. Two tumor specimens used in study I, one serous and one endometrioid tumor, turned out to be *BRCA1*- and *BRCA2* mutation-positive, respectively. One sporadic tumor which was used in study I was also used in study III as a sporadic reference case. Fifty-seven tumors (93%) exhibited DNA copy number changes. Gains were more common than losses, the ratio being 2.3:1. The most common gains affected 8q22-qter (54%). Other common regions of gain were, in descending order of frequency, 7q21-q34 (36%), 3q21-q26 (34%), 1q25-qter (30%), 2q32 (26%) and 6p22 (26%) (cut-off 25%). The most common high-level amplifications were detected at 8q22-q24.1 (18%), 3q26.1 (8%), 1q32 (8%), 6p21 (7%) and 12p12 (5%) (cut off 5%) (Table 3). The most common losses were found at 8p21-pter (26%), 18q12-qter (18%), 17p (16%), 13q22-q31 and 18p (15%) (cut-off 15%). Chromosomes 19 and 20 were excluded from the study I because some false-positive values occurred in the negative control experiments.

2. Sporadic carcinoma (I, III)

Forty-one sporadic carcinomas were studied using CGH analysis (Figure 3). Gains were more common than losses, their ratio being 2:1. On average, six aberrations per tumor were detected. The most frequent gain was found at 8q24.1-qter (54%). Other common gains occurred at 1q31-qter (29%), 7q21-q31 (29%) and 3q22-q26 (27%) (cut-off 25%). The most common losses were at 8p21-pter, 9p21-p23 and 17p12-pter (20%). Other frequent losses affected chromosomal regions 13q22-q31 (17%), 18q22 (15%) and 5q14 (15%) (cut-off 15%).

2.1. Serous carcinoma (I, III)

Combining the serous tumors from studies I and III, a total number of 26 tumors were studied. Gains predominated over losses at a ratio of 2.2:1. The most common gains were at 8q24.1-qter (65%), 7q32-33 (35%), 3q25-26.3 (35%), 1q32-qter (31%), 5p15 and 6p22-pter (27%). High-level copy number increases are shown in Figure 3. The most common losses affected 8p22-pter, 13q22-q31, 17p and 17q11.2-q21 (all 23%), 9p21-p23 and 18q22 (both 19%) and 4q, 5q , 6q25-qter and Xq (all 15%).

2.2. Mucinous carcinoma (I)

In mucinous carcinoma, the gain to loss ratio was 1.2:1. The most common region of gain was 17qcen-q21, found in 6 out of 8 tumors (75%). The most common loss was found at 9p in 3 out of 8 tumors (38%).

2.3. Endometrioid carcinoma (I)

In endometrioid carcinoma, gains were more frequent than losses, the ratio being 1.9:1. The most common gains affected 1q, in 4 cases out of 7 (57%), and 10q23-qter, in 3 cases out of 7 (43%). The most common losses occurred at 8p21-pter and 18p, in 2 out of 7 tumors (29%). One of the endo-

Table 3. High level amplifications in ovarian carcinoma detected by using CGH.

High level amplification	Number of cases/ cases studied	Reference
1q23-q32	3/ 24	Study I
1q32	1/ 20	Study III
1q32-qter	2/ 20 (BRCA2)	Study III
2p15-p22	1/ 24	Study III
2q22-q24	1/ 20	Study III
3qcen-q23	1/ 24	Study I
3q25-q26.1	3/ 20	Study III
3q26.1	2/ 20	Study III
3q27	3/ 25	Sonoda et al., 1997
5p	1/ 20 (BRCA1)	Study III
6p21	2/ 24	Study I
6p21.3	1/ 20	Study III
6p22-p24	2/ 20 (BRCA1)	Study III
6q13-qter	1/ 20	Study III
6q21-q22	1/ 20 (BRCA2)	Study III
7q36	2/ 25	Sonoda et al., 1997
8q	1/ 24	Study I
8q22-qter	4/ 20	Study III
8q23-q24.1	6/ 20 (BRCA1+2)	Study III
8q24.1-24.2	4/ 25	Sonoda et al., 1997
9p24	1/ 25	Sonoda et al., 1997
10p15	1/ 25	Sonoda et al., 1997
12p	1/ 20 (BRCA1)	Study III
12p12	2/ 25	Sonoda et al., 1997
12p12	2/ 20	Study III
12p12	9/ 47	Arnold et al., 1996
12q13-q21	1/ 20 (BRCA1)	Study III
12q14-q24.1	1/ 20	Study III
17q21-qter	1/ 20	Study III
17q25	2/ 25	Sonoda et al., 1997
18p11.3	1/ 25	Sonoda et al., 1997
20p	1/ 20 (BRCA1)	Study III

metrioid tumors included in study I revealed a *BRCA2* mutation.

2.4. Comparison of genetic changes among different histological subtypes (I, III)

Serous carcinoma exhibited more chromosomal changes than mucinous or endometrioid carcinomas, the average being 7.7 changes compared with 4.4 and 4.8 changes in endometrioid and mucinous tumors, respectively. Gains at 1q occurred in serous and endometrioid tumors only (Table 4). High-level copy number increases at 1q were common, especially in endometrioid tumors. Gains at 5p were observed only in serous carcinoma. Gains and losses at chromosome 6 were detected only in serous carcinoma, except for one mucinous carcinoma showing a gain at 6p. Losses at 8p were not found in the mucinous tumors and losses at 9p were not found in endometrioid carcinoma. Gains at chromosome 10 were not detected in mucinous tumors. Gains at 11q were predominantly found in serous tumors; only one endometrioid carcinoma showed a gain at 11q. Losses at 13q were common in serous carcinoma, whereas only one endometrioid carcinoma revealed a loss at 13q. Mucinous carcinoma did not show any losses at 13q. Losses at 17p were not found in endometrioid carcinoma. Gains at 17q were most frequent in mucinous tumors.

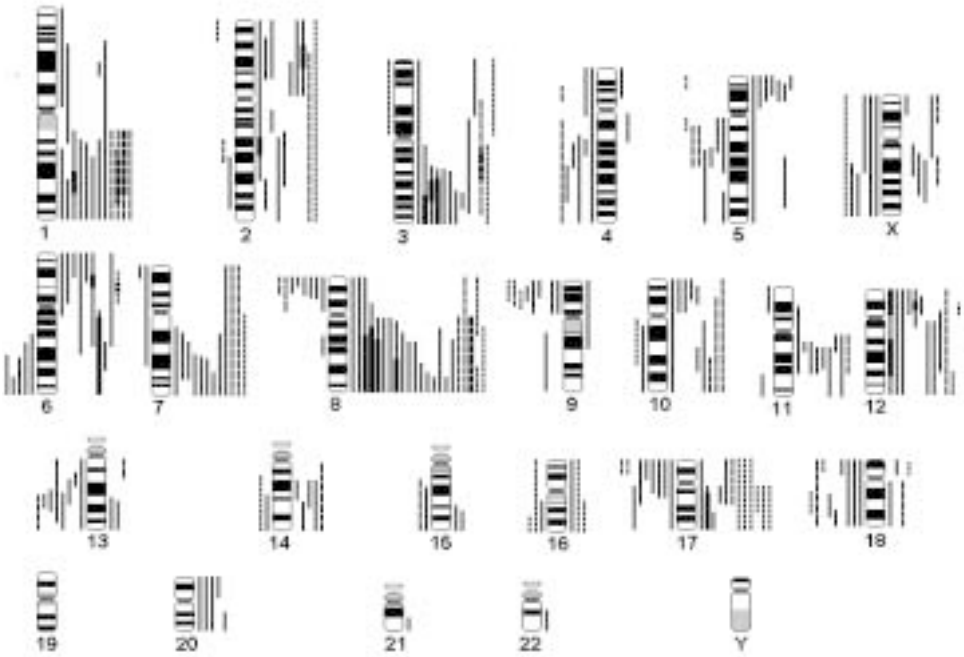

Figure 3. Summary of chromosomal gains and losses in 41 cases of sporadic ovarian carcinoma. Each line represents a change in one sample. Losses are displayed on the left and gains on the right of the chromosomes. High-level amplifications are shown bold. Serous tumors are marked with a continuous line, endometrioid tumors with a broken line and mucinous tumors with a dotted line.

Table 4. Comparison of the chromosomal changes between the serous, endometrioid and mucinous subtypes of ovarian carcinoma.

Chromosomal change	Serous	Endometrioid	Mucinous
+1q	8/26	4/7	0/8
+5p	7/26	0/7	0/8
+6p	7/26	0/7	1/8
-6q	5/26	0/7	0/8
-8p	6/26	2/7	0/8
-9p	5/26	0/7	3/8
+10p	5/26	2/7	0/8
+10q	3/26	3/7	0/8
+11q	8/26	1/7	0/8
-13q	6/26	1/7	0/8
-17p	6/26	0/7	2/8
+17q	4/26	1/7	6/8

3. Fallopian tube carcinoma (II)

Twenty fallopian tube carcinomas were analyzed by CGH. DNA sequence copy number changes were found in all cases. Gains were more common than losses, the ratio being 1.6 to 1. The most common gains were detected at 8q22-qter (75%) and 3q25-qter (70%). The other frequent gains occurred at 1q and 12p (40%), at 7q (35%), and at 5p and 20q (30%). High-level copy number increases were found at 8q22-qter (20%), 3q25-q28 (15%) and 12p (5%), in a total of six tumors. The most common losses affected 18q (35%), 8p and 5q (30%).

3.1. Comparison of genetic changes in serous ovarian, fallopian tube and endometrial carcinoma (II)

In serous carcinomas of the ovary, fallopian tube and endometrium the pattern of chromosomal changes was similar (Table 5). The most commonly gained genomic regions, i.e. 1q, 3q, 7q, 8q and 12p, and the most frequently lost regions, i.e. 4q, 6q, 8p, 13q and 18q, were similar in each cancer type. Loss at 17p occurred only in ovarian and fallopian tube carcinomas.

4. Inherited ovarian carcinoma (III)

Twenty cases of inherited carcinoma were screened for somatic genetic alterations by using CGH. Sixteen specimens were from patients with *BRCA1* mutations and four tumors were from patients with *BRCA2* mutations.

In the BRCA1 group, 13 of the 16 tumors exhibited DNA sequence copy number alterations (average 6 changes per tumor; SD 4.9) (Figure 4). Gains were three times as common as losses. Eight of the 16 tumors (50%) carried gains at 2q24-q32, 3q25-q26.3, 7q31 and 8q22-qter. Five tumors (31%) showed gains at 6p21.1-pter and 11q21. High-level copy number increases are displayed in Figure 4. The most common loss was at 8p22-pter, found in 6 tumors (38%).

In the BRCA2 group, all four specimens

Table 5. The most frequent chromosomal changes in serous ovarian, fallopian tube and endometrial carcinoma.

Chromosomal change	Ovary	Fallopian tube	Endometrium
+1q	30%	25%	25%
+3q	40%	70%	60%
+5p	30%	25%	35%
+6p	30%	25%	35%
+7q	35%	30%	25%
+8q	70%	75%	40%
+12p	30%	40%	20%
+20q	20%	30%	20%
-4q	20%	25%	15%
-6q	20%	15%	10%
-8p	25%	30%	10%
-13q	15%	20%	5%
-17p	25%	15%	0%
-18q	20%	30%	20%

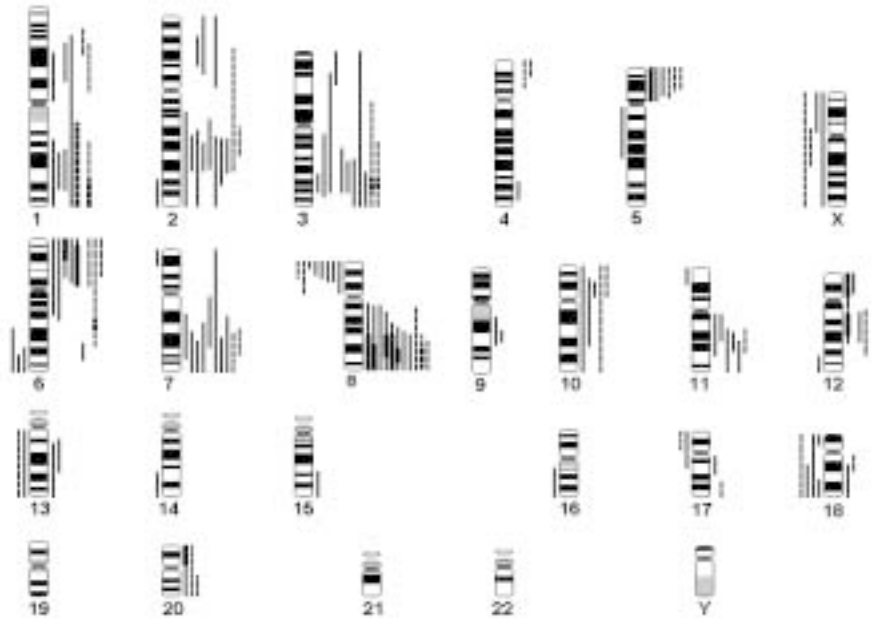

Figure 4. Summary of chromosomal gains and losses in 16 cases of *BRCA1*- and 4 cases of *BRCA2*-associated ovarian carcinoma. *BRCA1*-associated samples are marked with a continuous line and *BRCA2*-associated samples are marked with a broken line. Each line represents one sample. Losses are shown on the left and gains on the right of the chromosome. High-level amplifications are displayed bold.

showed DNA sequence copy number changes (Figure 4). Again, gains were more common than losses. The most common gains were found at 6p21.1-pter and 8q22-qter (three out of four cases, 75%). High-level copy number increases are shown in Figure 4. Losses were detected at 8p21-pter and Xq (in 2 out of 4 tumors; 50%).

4.1. Comparison of inherited and sporadic ovarian carcinoma (III)

Comparison of inherited and sporadic ovarian carcinoma revealed a similar pattern of chromosomal aberrations except for chromosomal gains at 2q24-q32 in BRCA1 mutation-associated tumors (Table 6). Gains at 2q24-q32 were more common in the BRCA1 mutation-associated serous cancers than in sporadic cancers.

5. Gene expression changes (IV)

5.1. Ovarian carcinoma versus benign adenoma (IV)

All six adenocarcinomas were compared with benign adenoma. Altogether, 59 differentially expressed genes were detected in adenocarcinoma. Thirty-eight of the genes were upregulated and 19 were downregulated. Sixteen genes were upregulated and nine were downregulated in all carcinoma samples. The upregulated genes included Rho family genes, oncogenes and tumor suppressor genes, genes for cell-cell interaction, invasion, cell fate and development, apoptosis regulators, cell cycle and growth regulators,

Table 6. Comparison of the frequency of the most common chromosomal changes in the *BRCA1*-mutation positive and sporadic ovarian carcinoma. NS, not significant.

Chromosomal change	BRCA1	Sporadic	Significance of the difference
+1q32-q41	25%	30%	P=1.00, NS
+2q24-q32	50%	15%	P=0.03
+3q25-q26.3	50%	40%	P=0.74, NS
-4q21-q24	0%	20%	P=0.11, NS
+5p14-p15.2	19%	30%	P=0.70, NS
+6p22-pter	31%	35%	P=1.00, NS
-6q25-qter	19%	20%	P=1.00, NS
+7q	50%	35%	P=0.35, NS
-8p23	38%	25%	P=0.48, NS
+8q24.1-qter	50%	70%	P=0.31, NS
+11q	31%	25%	P=0.72, NS
+12p11.2-p13	13%	30%	P=0.26, NS
-17p	6%	25%	P=0.20, NS
-18p11.2-pter	13%	20%	P=0.70, NS
-18q22-qter	19%	20%	P=1.00, NS

growth factors, receptors, and DNA damage repair. The downregulated genes included Wnt pathway genes, genes for DNA damage response and repair, invasion, angiogenesis, cell cycle and apoptosis regulators, receptors, intracellular kinase network members, and intermediate filament markers.

5.2. Local, highly differentiated carcinoma versus benign adenoma (IV)

A total of 49 genes were differentially expressed in local, highly differentiated carcinoma compared with benign adenoma. Twenty-three genes were upregulated and 26 genes were downregulated. The upregulated genes included those for regulators of apoptosis, cell-cell interaction, cell adhesion, motility and invasion, intracellular network members, invasion regulators, intermediate filament markers, and receptors. The downregulated genes included those for cell adhesion, motility and invasion, invasion regulators, Wnt pathway genes, growth factors, intermediate filament markers, cell-cell interaction, receptors and tyrosine kinases.

5.3. Advanced carcinoma versus local, highly differentiated carcinoma (IV)

Fifty-eight genes were differentially expressed in advanced carcinoma. In advanced carcinoma, 26 genes were upregulated and 32 were downregulated. Twenty-one genes were upregulated and 12 genes were downregulated in all advanced carcinomas. In this comparison the upregulated genes included cell adhesion genes, motility and invasion genes, receptor genes, oncogenes and tumor suppressor genes, genes for cell cycle and growth, cell fate and development regulators and rho family members, DNA damage response and repair genes, and cell-cell interaction genes. The downregulated genes included those related to apoptosis, intracellular kinase network members, cytokines and growth factors, DNA damage response and repair, angiogenesis and cell cycle and growth regulators, intermediate filament markers, invasion markers, receptors and cell fate and development regulators.

DISCUSSION

Not more than ten years ago, a standard cytogenetic technique was used as a screening method for chromosomal aberrations in cancer research. This technique is still very useful in studying hematological malignancies, but it has clear limitations in studying solid tumors, such as ovarian cancer. Chromosomal analysis requires cell culture, easily proliferating cells and good quality metaphase spreads. Cells from solid tumors do not grow well in cultures and the obtained metaphase spreads are often difficult to interpret. DNA-based methods have limited power in screening purposes because specific probes are needed for the analysis. These difficulties and limitations have led to the development of CGH. It was a first step towards the so-called array methods. CGH provides whole genome screening and mapping of chromosmal gains and losses. Knowledge of chromosomal aberrations in solid tumors has expanded rapidly (Knuutila et al., 1998; Knuutila et al., 1999). Even though CGH has proved its power in cancer research, it has certain limitations. It can provide only a rough copy number karyotype and, for example, translocations and small changes cannot be detected. Tumors consist of different cell populations and clones, and usually different parts of a tumor are not separated for the analysis. The aberrations detected in a tumor represent a "pool" of changes. This holds true for ovarian carcinoma. However, this can be avoided by tissue microdissection, in which specific parts or cells of the tissue can be recovered (Gonzalez et al., 1999; Luo et al., 1999). A recent development is the cDNA array technique, which enables large-scale and more detailed approaches. Arrays can contain thousands of cDNAs representing genes and ESTs. There are, however, certain requirements in using cDNA arrays. The quality and quantity of RNA have to be high. This requires careful handling of specimens. According to the manufacturers of the filter arrays, there is a risk of 10% false-positive gene expression signals. In addition, for data managment, sophisticated bioinformatic systems are needed.

1. Chromosomal gains and losses in ovarian carcinoma

On the basis of CGH analysis, ovarian carcinoma turned out to be genetically extremely complex. Only four carcinoma samples exhibited a normal CGH profile. Histopathological evaluation of the tumor samples confirmed that the samples consisted of more than 50% tumor cells. It is possible that the samples without any DNA sequence copy number changes still contained other aberrations, such as ploidy changes or balanced structural alterations, that cannot be visualized by the CGH method employed.

Nearly every chromosome was found to contain alterations. Clustering of the DNA copy number changes to certain chromosomal subregions was common (Figures 3 and 4). Some genomic regions with gains are known to contain oncogenes obviously playing a role in ovarian carcinoma. In the present study, many other regions with gains or losses were identified. In most of the deleted regions the genes of importance remain to be identified. In addition, CGH

revealed several highly amplified regions (Figures 3 and 4). These regions can be informative in the search for putative amplified oncogenes (Tanner et al., 1994).

In keeping with the results of other studies, the most prominent gains in ovarian carcinoma were detected at chromosome arms 1q, 3q, 7q and 8q (Table 2) (Iwabuchi et al., 1995; Arnold et al., 1996; Sonoda et al., 1997; Wolff et al., 1997; Blegen et al., 2000; Suzuki et al., 2000; Kiechle et al., 2001). Chromosomes 19 and 20 were excluded from study I because some false-positive values occurred in the negative control experiments. Chromosome 20, however, has shown consistant gains in other studies (Iwabuchi et al., 1995; Arnold et al., 1996; Sonoda et al., 1997; Wolff et al., 1997; Blegen et al., 2000; Suzuki et al., 2000; Kiechle et al., 2001). Gains at the telomeric part of chromosome arm 1q are also common in other cancers, but the candidate genes have not yet been identified (Knuutila et al., 1998). In solid tumors, changes at chromosome arms 3q and 8q have been associated with tumor progression (Isola et al., 1995; Arnold et al., 1996; Heselmeyer et al., 1996). Gain and high-level copy number increases at 3q have also been detected in many other cancers (Knuutila et al., 1998). There are obvious candidate genes at the telomeric part of chromosome arm 3q. Recently, the *PIK3CA* gene, located at 3q26, has been found to be amplified in ovarian cancer (Shayesteh et al., 1999). It encodes a subunit of phosphatidylinositol 3-kinase (PI3-kinase). Amplification of *PIK3CA* is associated with PI3-kinase activity. Many cancer-related functions, such as proliferation (Klippel et al., 1998), cell adhesion (Khwaja et al., 1997), apoptosis (Kennedy et al., 1997) and RAS signaling (Downward, 1998) have been associated with PI3-kinase-mediated signaling. Another possible candidate gene, at 3q26.3, is the human telomerase RNA gene (*hTR*), which encodes telomerase, an enzyme which maintains telomere length in the chromosomes. Telomeres are important for stabilizing the chromosomes during replication. Normal somatic cells undergo telomeric attrition in the absence of telomerase activity. It has been suggested that telomere shortening may act as a molecular clock (Holt et al., 1996). Telomerase activity has been linked to cellular immortality and tumor progression. Overexpression of telomerase has been found in ovarian carcinoma (Soder et al., 1997; Soder et al., 1998).

At chromosome arm 7q a common region of gain extended from band q21 to q33. A potent candidate gene at 7q31 is the *CMET* gene, which codes for a transmembrane tyrosine kinase receptor and is the ligand for hepatocyte growth factor/scatter factor. CMET is expressed in epithelial tissues, including normal epithelia of the female reproductive system (Huntsman et al., 1999). Increased expression of CMET has been found in ovarian carcinoma (DiRenzo et al., 1994; Huntsman et al., 1999). At chromosome arm 8q the smallest commonly gained region included bands q24.1-qter. However, many gains affected almost all of the long arm and in some cases displayed a profile with two peaks, one at 8q24.1-qter and another at 8q11-q21.1. High-level copy number increases, extending from band q12 to qter were common. A probable candidate gene at 8q24.1 is the *CMYC* oncogene. It is amplified in ovarian carcinoma (Zhou et al., 1988; Baker et al., 1990; Sasano et al., 1990) as well as in several other cancers (Bieche et al., 1995; Sato et al., 1999). CMYC is a transcription factor and it has a role in DNA replication (Ryan and Birnie, 1996). It is also known to induce apoptosis. However, the large size of the amplicons at 8q indicates that there may also be other important genes located at 8q.

The most notable losses were at 8p (26%), 13q (20%), 17p (16%) and 18q (18%). These results are in accordance with

those of previous and subsequent CGH studies in which the frequencies of losses have varied between 6 and 56% (Iwabuchi et al., 1995; Arnold et al., 1996; Sonoda et al., 1997; Wolff et al., 1997; Blegen et al., 2000; Suzuki et al., 2000; Kiechle et al., 2001). Variation of results between studies may be due to differences in study populations and small numbers of cases studied. Losses at 8p, the smallest common region narrowed down to q21-qter, have been detected in other tumors as well, especially in prostate cancer (Cher et al., 1994; Visakorpi et al., 1995). Loss at 8p has often occurred simultaneously with a gain at 8q, indicating isochromosome formation. Isochromosomes are thought to result from transverse division instead of longitudinal division of the centromere, or from translocation, or chromatid exchange involving two homologous chromosomes (de la Chapelle, 1982). Isochromosome formation at 8q has been found in other solid tumors as well (Castedo et al., 1991; Cher et al., 1994). At 8p, LOH is common in many solid tumors. In ovarian carcinoma, LOH at 8p has been found in 26–40% of cases (Cliby et al., 1993; Osborne and Leech, 1994). However, in these studies, fine mapping of 8p has not been feasible because of the limited number of polymorphic markers used. Use of a dense map of microsatellite markers has enabled the detection of a high rate of LOH at 8p22-p23 (Wright et al., 1998). Possible candidate tumor suppressor genes at 8p22 are *N33, MSR* and *FEZ1* (Cher et al., 1994; Bova et al., 1996; MacGrogan et al., 1996; Nihei et al., 1996; Ishii et al., 1999). The data suggest that 8p could harbor several tumor suppressor genes important in ovarian carcinoma.

The minimum common region of loss at 13q included bands q22-q31. The known tumor suppressor genes at 13q are *BRCA2* (q12) and *RB1* (q14.3), but they reside outside the lost segment. In ovarian carcinoma, LOH at 13q22-q31 has been reported in 23–30% of cases, but the number of cases and markers studied has been limited (Yang-Feng et al., 1993). In the present study, all losses at 13q, except for one, occurred in serous carcinoma. This is accordance with the results of earlier LOH studies in which 13q losses were related to serous histology (Sato et al., 1991). Recently, 13q21-q22 has been reported to show frequent LOH in carcinoma of the prostate (Hyytinen et al., 1999). It is likely that there are still unknown tumor suppressor genes at 13q that may play a role in ovarian carcinoma.

Loss of the *TP53* gene, located at 17p13.1, possibly explains most of the 17p losses. However, two interesting candidate genes, *OVCA1* and *OVCA2*, assigned to 17p13.3, may also contribute to the losses at 17p. Decreased mRNA expression of *OVCA1* and *OVCA2* have been reported in ovarian carcinoma (Schultz et al., 1996). Chromosomal region 18q21-q23, commonly deleted in ovarian and in fallopian tube carcinoma, includes the well characterized tumor suppressor genes *DCC* and *Smad4*. The *DCC* gene encodes netrin receptor (Keino-Masu et al., 1996). Netrin is a chemoattractant needed for guidance of developing axons in the nervous system (Keino-Masu et al., 1996). Inactivation of DCC has been found in several malignancies, such as colorectal (Fearon et al., 1990), breast (Devilee et al., 1991), prostate (Gao et al., 1993), and pancreatic cancers (Hohne et al., 1992). However, in ovarian carcinoma, LOH has been found at 18q21, but not at the *DCC* locus within the same region. *Smad4* is a member of the *Mad* gene family. The *Smad* genes are involved in signal transduction between TGF beta receptors and the cell nucleus. *Smad4* inactivation occurs in about 50% of pancreatic carcinomas but is uncommon in other tumors, including ovarian cancer (Hahn et al., 1996; Schutte et al., 1996; Takakura et al., 1999).

2. Comparison of chromosomal alterations among the different histological subtypes

The present results suggest that serous ovarian carcinoma displays more chromosomal alterations than mucinous or endometrioid carcinoma. This is in accordance with the observation that, using conventional cytogenetic analysis, abnormal karyotypes are more frequent in serous carcinoma (Pejovic et al., 1992). In CGH analysis, different histological subtypes of ovarian carcinoma exhibit different genomic alterations. Differences were detected at 1q, 5p, 6, 8p, 9p, 10, 11q, 13q and 17. Of the known oncogenes, *INT2* and *ERBB2* are located at 11q13 and 17q12, and the tumor supressor genes *D0C2, MTS1* and *TP53* at 5p13, 9p21 and 17p13, respectively. Molecular genetic analyses of *TP53* (Klemi et al., 1995), *KRAS* (Enomoto et al., 1990; Mok et al., 1993; Ichikawa et al., 1994), *BCL2* (Diebold et al., 1996) and *D0C2* (Mok et al., 1998) have indicated genotypic differences between various histological subtypes of ovarian carcinoma. In LOH analysis, changes at chromosome arms 6q, 13q and 19q have been linked to serous histology (Sato et al., 1991). The present results suggest that the different histological subtypes of ovarian carcinoma may have distinct genetic pathways during carcinogenesis and tumor progression. However, in CGH studies the numbers of endometrioid and mucinous tumors have been too small to allow any firm conclusions to be made (Iwabuchi et al., 1995; Arnold et al., 1996; Sonoda et al., 1997; Wolff et al., 1997; Blegen et al., 2000; Suzuki et al., 2000; Kiechle et al., 2001).

3. Chromosomal gains and losses in fallopian tube carcinoma and comparison with changes in serous ovarian and endometrial carcinoma

The genetic background of fallopian tube carcinoma is poorly understood. Molecular genetic analyses concerning *TP53*, *ERBB2* (Lacy et al., 1995) and *KRAS* (Mizuuchi et al., 1995) in this tumor type have been carried out, but only a few cytogenetic studies have been reported (Bardi et al., 1994). In studies using CGH, fallopian tube carcinomas have shown extensive genomic alterations. As in ovarian carcinoma, the most common gains were at loci 3q and 8q and the most frequent losses were at 8p and 18q. The present results agree with those of Heselmeyer and co-workers (1998), who studied 12 primary fallopian tube carcinomas and found the most frequent alterations to be gains at 1q, 2q, 3q and 8q, and losses at 8p and 18q. They also found common losses at 16q and 22q. In the present study, frequent losses in these regions were not detected. The discrepancy between the two studies may involve the sensitivity of chromosome 16 as regards artefacts, which may lead to false ratio values. The relatively small number of tumor samples analyzed in both studies could also explain the variation in the results.

Fallopian tube carcinoma also shared other changes compared with serous ovarian carcinoma. The main pattern of aberrations resembled that found in serous uterine carcinoma (Pere et al., 1998). However, the changes were different from those seen in other histological subtypes of ovarian and uterine carcinoma (Pere et al., 1998). Clinically, serous carcinomas of the ovary, fallopian tube and endometrium exhibit invasive behavior, early dissemination and are associated with poor survival (Blaustein, 1982). Histopathologically they are similar. *TP53* mutations are frequent in all these tumors (Klemi et al., 1995; Lacy et al., 1995; Sherman et al., 1995; Caduff et al., 1998). To summarize, these similarities suggest that serous carcinomas of the ovary, fallopian tube and endometrium may have a common genetic pathway during tumor development and progression.

4. Chromosomal gains and losses in inherited ovarian carcinoma and comparison with the changes in sporadic ovarian carcinoma

In addition to genetic predisposition, i.e.,germ-line mutation of the *BRCA1* or *BRCA2* gene and loss of the corresponding wild-type allele, somatic genetic changes are required for the malignant phenotype to develop. In this study, somatic genetic changes were screened by CGH. Inherited ovarian carcinoma exhibited complex somatic genomic changes, so that almost every chromosome appeared to contain genomic abnormalities. Clustering of the changes to certain chromosomal regions was observed. The genomic abnormalities resembled those found in sporadic serous ovarian carcinoma, both qualitatively and quantitatively. The most prominent gains affected the same genomic subregions as in sporadic carcinoma, except for the gain at chromosome arm 2q. Gains at 2q, the smallest common region being 2q24-q32, were more frequent in *BRCA1*-associated carcinoma compared with sporadic carcinoma. The significance of the 2q gain is, however, obscure. An interesting gene at 2q34-q35, which is distal to the minimum common region, is *BARD1* (BRCA1-associated ring domain 1). BARD1 shares homology with two conserved regions in the BRCA1 protein (Wu et al., 1996). It interacts with BRCA1, and is essential for BRCA1-regulated tumor suppression (Wu et al., 1996). In the 2q24-q32 region there are many possible candidate genes, but none of them has been studied so far by targeted molecular genetic analyses in ovarian carcinoma.

The *BRCA1*- and *BRCA2*-associated ovarian carcinomas show mainly serous histology (Boyd and Rubin, 1997). The similarity of the genomic changes in inherited and sporadic serous ovarian carcinomas suggests that serous ovarian carcinoma, irrespective of its etiology, may follow a common main pathway during tumor progression.

5. Gene expression changes in serou ovarian carcinoma

The cDNA array analysis of serous ovarian carcinoma uncovered many genes with an altered expression level. Some of the changes found in this study, e.g. upregulation of *PCNA* (Barboule et al., 1998), *CMET* (DiRenzo et al., 1994) and *AKT2* (Bellacosa et al., 1995), have been reported earlier in ovarian carcinoma. Our results thus confirm earlier ones and provide further evidence of the reliability of the cDNA array method employed. Previous large scale expression analyses of ovarian carcinoma (Wang et al. 199; Ono et al., 2000) and ovarian carcinoma cell lines (Ismail et al., 2000) have also revealed differences in many genes. However, these studies cannot be directly compared. In two previous studies of ovarian carcinoma the reference sample has been normal ovarian tissue. In the present study, normal ovarian tissue was not included in the analysis. In addition, the precise cDNA contents of the arrays used in other studies are not known.

In the present study, the most prominent differences between benign adenoma and carcinoma were upregulation of *RH0GDI2*, *CMET* and *DLG3*, and downregulation of *HGFAC*, *DES* and *PDGFRA*. The *RH0GDI2* gene was upregulated in all cases of carcinoma, irrespective of tumor stage, suggesting that this gene may be essential as regards serous ovarian carcinogenesis. The *RH0GDI2* gene acts as a regulator in the guanine nucleotide di/tri phosphate (GDP/GTP) cycle, and in cell membrane association/dissociation of Rho proteins (Olofsson, 1999). The *RH0GDI1* and *Rho 8* genes were also upregulated. The Rho family of proteins are components in cellular processes which control organization of the actin cytoskeleton, activate kinase cascades, regulate gene expression, promote growth transformation and induce apoptosis (Zohn et al., 1998). However, the role of *RH0GDI2* in human carcinogenesis is unknown. The

RH0GDI2 gene has not been linked to ovarian carcinoma before.

Comparison of local, highly differentiated adenocarcinoma and benign adenoma also revealed that the most highly upregulated gene was that for the cell division cycle 42 homolog (*cdc42*). This gene was also upregulated in advanced carcinoma, but at a lower level than in local carcinoma. It has been suggested that *cdc42* can alter regulation of normal cell growth and initiate tumorigenic signals (Lin et al., 1999). It is of interest that cdc42 is a Rho-related GTP-binding protein. It may represent another mechanism by which the Rho-pathway can play a role in the development and progression of serous ovarian carcinoma. There are no previous reports of upregulation of cdc42 in ovarian carcinoma.

The most frequently downregulated gene in ovarian carcinoma was that for hepatocyte growth factor activator (*HGFAC*). It was downregulated in all carcinoma samples, but the downregulation was not as prominent in local, highly differentiated carcinoma as in advanced cancers. HGFAC is a serine proteinase that activates an inactive, single-chain form of hepatocyte growth factor (HGF). HGFAC is the key enzyme that regulates the activity of HGF in injured tissues (Miyazawa et al., 1996). In glioblastoma, simultaneous expression of *HGF*, *HGFAC* and the HGF receptor gene *CMET* has been detected (Moriyama et al., 1995). The downregulation of *HGFAC* in ovarian carcinoma has not been reported before.

In this study, the most obvious differences between advanced and local carcinoma were upregulation of *C0L3A1*, *FGFRI* and *CMET*, and downregulation of *HGFAC*, *FZD3* and *BFL1*. The gene for collagen type III alpha 1 (*C0L3A1*) was upregulated in all three advanced carcinomas. The protein is a component of soft connective tissue. It has been suggested that progressive ovarian carcinoma may induce expression of type III procollagen both in the tumor tissue and in the peritoneal cavity (Zhu et al., 1993). In addition, in poorly differentiated serous ovarian carcinoma, the formation of type III procollagen may occur in the neoplastic cells (Kauppila et al., 1996). In serous ovarian carcinoma, production of type III procollagen has been found to be related to an increased degree of malignancy (Kauppila et al., 1996). In breast cancer, the increased synthesis of type III procollagen has been suggested to play a role in tumor invasion (Kauppila et al., 1998). Thus, it is possible that upregulation of type III collagen may also be involved in the progression of serous ovarian carcinoma.

The present study showed differences in gene expression between benign and malignant serous ovarian tumors, and between local and advanced serous adenocarcinomas. The limited sample size does not allow firm conclusions. It can be speculated, however, that the differentially expressed genes found in malignant tumors may be associated with carcinogenesis, whereas those genes which are differentially expressed in advanced but not in local carcinoma may play a role in tumor progression.

6. The clinical significance of chromosomal changes

The clinical significance of chromosomal gains and losses is still unclear. Tumors with poor histological differentiation (grade) have exhibited more changes than highly or moderately differentiated tumors (Iwabuchi et al., 1995). A high number of aberrations, the presence of the most common changes, e.g. gains at chromosomal arms 3q and 8q, and loss at 16q, have been associated with poor outcome (Iwabuchi et al., 1995; Suzuki et al., 2000). Some alterations, such as gains at 3q24-qter, 7p, 8q24 and 20q13.2-qter, and losses at 11p and 13q, have been found to be common in advanced stage and/or poorly differentiated tumors, suggesting that these changes are associated with dis-

ease progression (Iwabuchi et al., 1995; Sonoda et al., 1997; Kiechle et al., 2001). In contrast, Suzuki and co-workers (2000) have reported that gains at 3q, 8q and 20q are common in well differentiated tumors. To the list of changes in well differentiated tumors can be added gains at 12p and 18p (Kiechle et al., 2001).

Kudoh and co-workers (1999) studied cisplatin resistance in ovarian carcinoma and concluded that gains at 1q21-q22 and 13q12-q14 may be indicators of drug resistance. This finding is different from that regarding cisplatin-resistant ovarian carcinoma cell lines, in which the critical chromosomes have been reported to be 2, 4, 6, 7, 8, 11, 13 and X (Wasenius et al., 1997). Consistent correlations between genetic aberrations and clinical behavior have not been demonstrated, probably due to the limited number of tumors studied, the genetic complexity of the tumors and the wide spectrum of their biological properties.

The findings of the present study do not have straight clinical correlations or applications. However, the identification of specific genetic changes in different histological subtypes of ovarian carcinoma may be an important step on the road to development of more individual cancer therapy. The similarity of the changes detected in serous carcinomas of the female reproductive organs, i.e. ovary, fallopian tube and endometrium, may be of help in understanding the clinically similar behavior of these cancers. In general, the genetic changes linked to ovarian carcinoma can help us to find new prognostic markers.

SUMMARY AND CONCLUSIONS

The aim of the present study was to investigate the molecular cytogenetic and molecular genetic changes in ovarian carcinoma. Comparative genomic hybridization (CGH) analyses were performed on both sporadic and inherited ovarian carcinomas, including different histological subtypes. The study was further extended to serous carcinomas of the fallopian tube and the endometrium to clarify the genetic relationship between serous carcinoma of the different reproductive organs. In addition, a large scale survey of gene expression changes was performed in serous ovarian carcinoma of different stages and histological grades.

Comparative genomic hybridization revealed complex chromosomal aberrations in ovarian carcinoma. Almost all chromosomes showed abnormalities. Chromosomal gains predominated over losses. Clustering of the gains to chromosomal arms 1q, 3q, 7q and 8q, and losses at arms 8p, 13q, 17p and 18q were detected. The most frequent high-level amplifications occurred at chromosomal bands 1q32, 3q26.1, 6p21, 8q22-q24.1 and 12p12.

In the CGH analysis, the most common histological subtypes (serous, mucinous and endometrioid) of ovarian carcinoma displayed distinct changes. This suggests that different histological subtypes may have different pathways of tumor development and progression. When comparing serous carcinoma of the ovary, fallopian tube and endometrium, the most common genomic changes affected the same chromosomal regions in all cancers. It is thus possible that serous carcinoma of the ovary, fallopian tube and uterus share genetic changes needed for tumor evolution. Sporadic serous ovarian carcinoma and *BRCA1*- and *BRCA2*-associated ovarian carcinoma, which are also of serous histology, displayed strikingly similar genomic changes, except for gains at chromosome arm 2q. Gains at 2q24-q32 were more frequent in *BRCA1*-associated carcinoma than in sporadic carcinoma. This indicates that serous carcinoma of the ovary, despite the etiological differences in sporadic and inherited tumors, may follow a common pathway during tumor progression.

The cDNA array analysis uncovered differences in gene expression between benign and malignant serous ovarian tumors, and between local and advanced serous carcinomas. It is possible that the differentially expressed genes found in this study are associated with carcinogenesis and tumor progression.

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