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„Establishment and molecular characterisation of high grade serous epithelial ovarian cancer cell lines“

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STATUTORY DECLARATION

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Zusammenfassung

Das Ovarialkarzinom, oder auch Eierstockkrebs, welches jährlich rund 225 000 Fälle betrifft, zeigt eine erschreckende Todeszahl von zirka 140 000. Das epitheliale Ovarialkarzinom ist der Typ von Eierstockkrebs der jährlich die höchste Todesrate aufweist. 75% der Patientinnen erkranken am hochgradigen serösen Ovarialkarzinom bei dem fast alle Patientinnen eine Mutation im *TP53* Gen aufweisen.

Da es keine effizienten, spezifischen Tests gibt und der Verlauf der Krankheit bei Eierstockkrebs sehr asymptomatisch ist, kann die Krankheit in den meisten Fällen erst in einem sehr fortgeschrittenen Stadium diagnostiziert werden. Unter den Standardtherapien zählen zum einen, der operative Eingriff, bei welchem versucht wird den kompletten Tumor zu entfernen und zum anderen, die platinhaltige Chemotherapie. Obwohl es vielen Patientinnen nach dem Eingriff wieder gut geht, erleiden einige die Wiederkehr eines resistenten Ovarialkarzinoms, welches oft tödlich endet. Dies erklärt auch die niedrige 5 Jahres Überlebenschance beim hochgradigen serösen Ovarialkarzinom von etwa 30%.

Eine kleine Untergruppe an Tumorzellen im Primärtumor könnte für die Wiederkehr und die Chemo-resistenz der Zellen verantwortlich sein. Um die molekularen Mechanismen dieser Krankheit zu untersuchen und dieser Hypothese nachzugehen, werden dringend experimentelle Modelle benötigt. Das Ziel dieser wissenschaftlichen Arbeit war es, primäre Zellkulturen zu etablieren, welche als repräsentativ für das hochgradig seröse Ovarialkarzinom gelten und für die Forschung am Ovarialkarzinom unerlässlich sind.

Aszites und Tumorgewebe von insgesamt 24 Patientinnen wurde als Primärzellkultur angesetzt. *TP53* Mutationen im Tumorgewebe wurden mittels Hefe-Assay detektiert. Spezifischen *TP53* Mutationen wurden als Tumormarker für die Kontrolle und die Identifizierung in der Zellkultur eingesetzt. 13 primäre Zellkulturen und Zelllinien von insgesamt 11 Patientinnen konnten etabliert werden. Mittels *TP53* Mutations- und Proteinexpressionsanalysen wurden zwei unterschiedliche Zelltypen identifiziert. Der erste Typ von Zellen stellt die Tumorzellen aus dem Tumorgewebe dar. Die zweite

Zellpopulation stellt mit einer repräsentativen Wahrscheinlichkeit einen Typ dar, der von Mesothelzellen aus vom Peritoneum abstammen.

Zusammenfassend wurden primäre Zellkulturen und Zelllinien vom hochgradig serösen Ovarialkarzinom etabliert, welche in Zukunft als wichtige Modelle in der Forschung an dieser schweren Krankheit beitragen können.

Abstract

Ovarian cancer has an incidence of about 225000 women worldwide and causes approximately 140000 deaths annually. Epithelial ovarian cancer is the most lethal type of ovarian cancer. 75% of the patients present high grade serous ovarian cancers, almost all of them harbouring a *TP53* gene mutation. Due to the lack of efficient screenings and the asymptomatic course of the disease, many patients are diagnosed at an advanced stage of disease. Standard treatment includes surgery, in which tumour mass is removed maximally, and platinum-based chemotherapy. Even though most patients can achieve complete remission, most of them relapse with resistant disease and eventually die. The 5-year survival rate of high grade serous ovarian cancer patients is below 30%.

It has been suggested that a small sub-population of cancer cells within the primary tumour is responsible for the recurrent disease and chemoresistance. In order to study the molecular mechanisms, experimental models are urgently needed. The objective of this work is to establish primary cell culture and cell lines, which can represent the high grade serous ovarian cancer and be used for ovarian cancer research.

Ascites and tumour tissues from a total of 24 patients were set up as primary culture. *TP53* mutations in tumour tissues were determined using a functional yeast assay. The specific *TP53* mutation was used as a tumour marker to monitor the cell culture. 13 primary cell culture and cell lines could be established from 11 patients. Analyses of *TP53* mutation and protein expression revealed two different cell types. While one type represents the tumour cells in tumour tissue, the other one might be derived from mesothelial cells in the peritoneal cavity.

In conclusion, primary cell culture and cell lines representing high grade serous ovarian cancer have been established, which can serve as models to further study this disease.

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Introduction

Ovarian cancer

Today, cancer is sadly a leading cause of death and annually, around 7.6 million people die of cancer worldwide. The incidence of ovarian cancer is around 225000 and approximately 140000 women die of this disease.[1]

The majority of ovarian cancers are epithelial ovarian cancers (EOC), which accounts for 4% of cancer deaths in women (Jemal, Siegel et al. 2010). More than 75% of patients with EOC are diagnosed at an advanced stage of disease. Standard therapy includes surgery and chemotherapy. Although most of the patients show complete clinical remission after the first line treatment, most of them will relapse and develop resistant disease which eventually causes death. Together with the lack of screening and no early detection methods, the asymptomatic course of the disease, the recurrence of the disease and the resistance of the tumour to chemotherapy results in a very poor long term survival for ovarian cancer patients (Sueblinvong, Ghebre et al. 2012). Over the past 20 years, despite improvements in debulking surgery, in which the tumour is maximally removed and the combination of platinum and paclitaxel-based first-line chemotherapeutic regimens (Vaughan, Coward et al. 2011), the 5-year survival period is only 44% for all types of ovarian cancer and lower than 30% in EOC (Baldwin, Huang et al. 2012).

High grade serous ovarian cancer (HGSOC) is the most frequent histological type of epithelial ovarian cancer. Others include mucinous, endometrioid, and clear cell cancers. Over the past 20 years, EOC was believed to develop from malignant transformations of the ovarian epithelium (Cannistra 2004). Recent publications suggest that HGSOC arises from non-ovarian tissues, most probably from the fallopian tube (Vaughan, Coward et al. 2011, Kessler, Fotopoulou et al. 2013). The mucinous invasive ovarian cancer might originate as metastasis from the stomach or the colon-appendix. The endometrioid and the clear cell ovarian cancer could develop through retrograde menstruation from the endometrium.

Ascites is a gastroenterological term for an accumulation of fluid in the peritoneal cavity which is present in many cases of ovarian cancer patients. It can vary from a few dozen

[1] http://www.who.int/; GLOBOCCAN 2008, globocan.iarc.fr [2013/10/28]

millilitres up to many litres. The reason for fluid accumulation in ovarian cancer patients remains poorly understood (Puiffe, Le Page et al. 2007). The cellular fraction of ascites in ovarian cancer patients consists of cancer cells in form of single cells or spheroids, lymphocytes as part of the immune reaction and mesothelial cells from the peritoneal cavity which are released due to cancer (Burleson, Casey et al. 2004).

Histopathological characteristics of ovarian cancer

Histological Types of EOC

According to the Federation of Gynaecology and Obstetrics (FIGO) and the World Health Organisation (WHO), there are four major histological types of EOC: serous, mucinous, endometrioid and clear cell ovarian cancer (Serov and Scully 1973).

FIGO Stages of Ovarian Cancer[2]

Stage I (early disease) up to Stage IV (advanced disease) are defined for ovarian cancer based on the spread of tumour growth. Additionally, each FIGO stage is again sub-classified as A, B or C according to the detailed location of tumour occurrence.

Stage I - Growth of the cancer is limited to the ovary or ovaries (IA, IB, IC).

Stage II - Growth of the cancer involves one or both ovaries with pelvic extension (IIA, IIB, IIC).

Stage III - Growth of the cancer involves one or both ovaries, and one or both of the following are also present: (1) the cancer has spread beyond the pelvis into the lining of the abdomen; and (2) the cancer has spread to lymph nodes. The tumour is limited to the lesser pelvis but with histologically proven malignant extension to the small bowel or omentum (IIIA, IIIB, IIIC).

Stage IV - This is the most advanced stage of ovarian cancer. Growth of the cancer involves one or both ovaries and distant metastasis (the spread of the cancer to organs

[2] http://www.ovarian.org/types_and_stages.php [2013/10/14]

located outside of the peritoneal cavity) have occurred. Finding ovarian cancer cells in pleural fluid (from the cavity which surrounds the lungs) is also an evidence of stage IV disease.

Tumourigenesis

Genetic alterations of cells play a decisive role in tumourigenesis. Many genes have been involved in the occurrence and progression of cancer. Tumour suppressor genes and oncogenes, such as *TP53* and *KRAS*, play very crucial roles in cell proliferation and cell cycle. Genetic mutations of these genes can affect the normal biological processes of the cells and thus cause the malignancies.

Two different pathways were described for tumourigenesis of epithelial ovarian cancers designated Type I and Type II. Type I includes mucinous carcinoma, endometrioid carcinoma, malignant Brenner tumours and clear cell carcinoma. They are frequently associated with mutations in *BRAF*, *KRAS* and *PTEN* gene. Type II EOC is mostly related to high grade serous ovarian cancer. Almost all of them have mutations in the *TP53* gene (Shih Ie and Kurman 2004).

Tumour recurrence

Standard treatment for all ovarian cancers is maximal cytoreductive surgery, where as much as possible of the tumours is removed, followed by platinum-based chemotherapy. Even though most patients can achieve complete clinical remission after this first –line treatment, most of them relapse with resistant disease which eventually causes death.

It has been suggested that a small sub-population of cancer cells within the tumours have unique molecular characteristics that enable them to escape from the very toxic chemotherapy and later grow to recurrent disease. Others hypothesize that some cells in the primary tumours will acquire the resistance under the circumstances of first-line chemotherapy.

In the literature, these types of tumour cells which cause tumour recurrence and are resistant to chemotherapy are often described as "stem cells". Under this general term, variants of the "stem cell" hypotheses have been proposed with supports of different experimental data. Figure 1 shows one of the hypotheses.

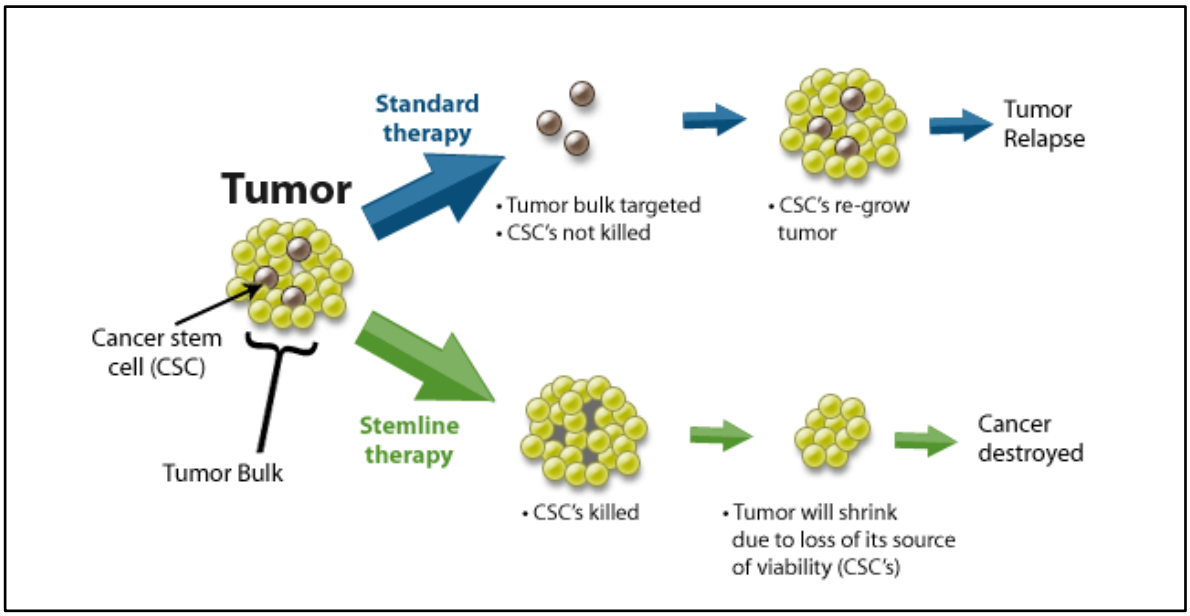

Figure 1. Schematic presentation of one of the tumour stem cell theories. Cancer stem cells with self-renewal ability are shown in grey colour, while the tumour cells constructing the tumour bulk are shown in yellow. Platinum-based standard therapy kills the "normal" tumour cells and leaves the tumour stem cells alive, which will develop to recurrent tumours under favourable conditions. If new therapy (stem line therapy) against "tumour stem cells" can be developed, they will kill the self-renewable tumour cells so that the tumour will be eventually destroyed by losing its capacity to grow[3].

So far, this small population of cells is not well identified and characterized. If key molecules or pathways for tumour resistance and recurrence can be identified and validated, they could be targeted by new therapeutic drugs that can be applied together with platinum-based chemotherapy to eliminate tumours. Finally, the survival of the patients can be prolonged and the patients can be cured (Kwon and Shin 2013).

In some types of cancers, for example breast cancer, distant metastasis is considered to take place by epithelial mesenchymal transition (EMT; Figure 2). In this case, cancer cells that have epithelial characteristics in the first place, gain the characteristics of mesenchymal cells which enable them to enter the circulatory system and to nest in

[3] http://breastcancerbydrruddy.com/wp-content/uploads/2011/05/Cancer-Stem-Cell.gif [2013/08/10]

distant organs. Distant metastasis in ovarian cancer is unusual both at primary diagnosis and during tumour progression.

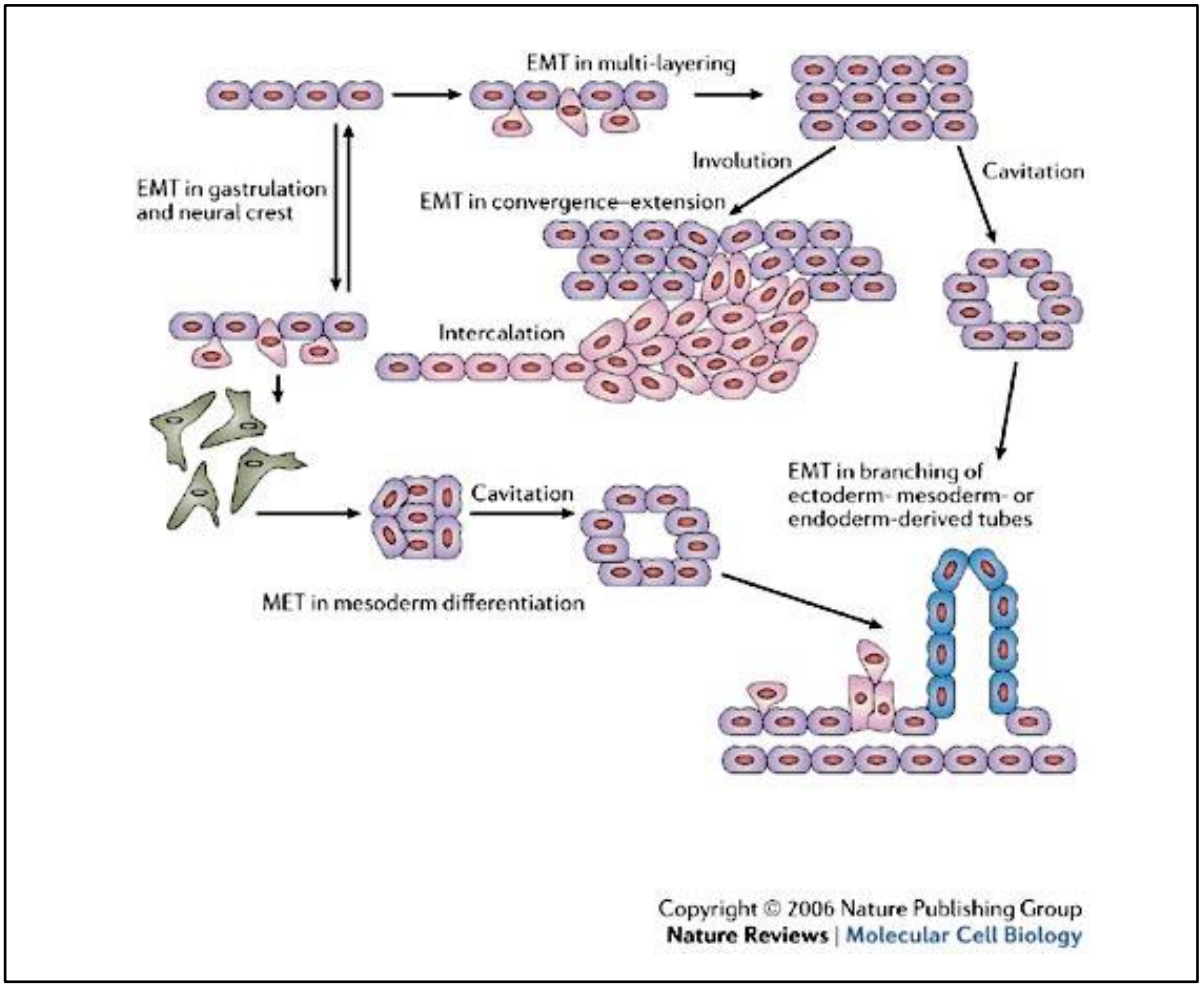

Figure 2. Schematic depiction of epithelial mesenchymal transition (EMT); (Thiery and Sleeman 2006).

Mutations in the *TP53* gene

TP53 mutations are one of the most frequent genetic alterations in human cancer and occur in around 50% of all malignant tumours. Most mutations are single-base substitutions with predominating missense mutations which are distributed throughout the coding sequence. *TP53* mutations can be potential prognostic and predictive markers, as well as targets for pharmacological intervention (Olivier, Hollstein et al. 2010). The majority of these mutations are located in the DNA-binding domain. Contact mutants affecting codons C248 and C273 interact directly with the DNA helix in different ways (Olive, Tuveson et al. 2004), (Song, Hollstein et al. 2007). Structural mutants

affecting the codons C175, C220, C245, C249 and C282 interact with the overall architecture of the DNA-binding surface and change the conformation of the protein. It was shown that mouse models were extremely tumour-prone when expressing mutant $TP53^{C172}$, corresponding to human C175 (Lang, Iwakuma et al. 2004). Mutations in other gene regions such as the transactivation domain, the proline-rich domain, the tetramerisation domain and the C-terminal region affect p53 regulation and are important for its activity but are extremely rare in human cancers. Some groups have shown that the tetramerisation domain and the C-terminus region are necessary for the gain of function by p53 mutants (Chene 2001), (Chene and Bechter 1999), (Mateu and Fersht 1998).

In a study, patients with *TP53* mutations in highly conserved domains, such as the DNA binding domain, had a shorter disease free interval and overall survival compared to those with mutations in non-conserved domains or wild type p53 (Reles, Wen et al. 2001). In addition, overexpression of p53 or *TP53* missense mutations showed more frequent resistance to adjuvant cisplatin or carboplatin chemotherapy (Reles, Wen et al. 2001). In another study *TP53* mutations were associated with distant metastasis in ovarian cancer patients (Sood, Sorosky et al. 1999). It was shown that tumours with null mutations were more likely to have metastatic spread to the lymph nodes than those with missense mutations or wild-type p53.

Experimental models to study ovarian cancer

Cell lines

High grade serous ovarian cancer is a major killer. In order to study the molecular mechanisms underlying this cancer, model systems representing the HGSOC are required. However, to date, there are no optimal model systems, a situation which needs to be improved urgently (Vaughan, Coward et al. 2011).

For decades, cell lines have been used as in vitro ovarian tumour models, from which most of our knowledge of ovarian cancer has been obtained, especially those regarding the functional molecules and pathways involved in the disease. Recently, a systematic genomic analysis has been performed on a panel of 47 ovarian cell lines in comparison

with thousands of tumour samples. Evidences on copy-number variant, mutation status and mRNA expression profiles reveal pronounced differences between the cell lines and the tumour tissues, suggesting that many of the most commonly used "ovarian cancer" cell lines most unlikely originate from the high grade serous ovarian cancer and thus are not proper models for studying the disease (Domcke, Sinha et al. 2013). Astonishingly, thousands of publications on ovarian cancer have been generated from results using cell lines such as SKOV3 or A2780, two lines that have no mutant p53 and are most unlikely of HGSOC origin. Among the 47 cell lines there are only a few which are most likely to be HGSOC, from which very few publications are generated.

In addition to the histological type, most of the currently used cell lines are not generated from resistant tumours. By inducing tumour resistance *in vitro*, over dosage of drugs are often used, which may cause additional genomic alterations of the cells making them not representative as they really are *in vivo.* So far, there was only one report on the establishment of a paired cell lines from the same patient, one of which represented a model of resistance acquired during the treatment of the patient (Robson, Lewis et al. 1987). However, there was no documentation of the histological type of the patient. Neither was there the status of *TP53*.

Xenograft and animal models

Xenograft models have been used for ovarian cancer research. Some genetically engineered mice models with oncogenic changes have also been reported (Sale and Orsulic 2006). The first xenograft model was created by implanting human epithelial ovarian cancer cell line cells into nude mice (Ward, Wallace et al. 1987). Today, these models are often used for testing therapeutic effects and studying the micro-environment of cancer (El-Gazzar, Perco et al. 2010). Xenografts could be a suitable model to study the effects of drugs on tumours. However, they have to reflect the originating cells and the underlying genomic events driving the disease. In particular, HGSOC should be recapitulated, which is unfortunately still not reached by the state of the art.

Besides xenografts, there were also approaches on establishing models in immune competent mice (Roby, Taylor et al. 2000). Mouse ovarian surface epithelial cells were isolated and cultivated for more than 20 passages and were shown to be tumourigenic *in vitro*. These cells were injected subcutaneously or into the peritoneum that induced

formation of tumours and production of ascites. This is a very unique model to study ovarian cancer. However, further investigations will be needed to explore its genetic and molecular association with HGSOC.

Another group has developed transgenic mice deficient for *TP53* and two of other three oncogenes *c-Myc, KRAS* and *AKT1*. By injecting these cells at subcutaneous, intraperitoneal or ovarian sites, tumours could be induced (Orsulic, Li et al. 2002). The tumours were demonstrated to resemble human ovarian carcinomas. However, multiple deficiencies of genes in somatic cells do not represent the genomic events occurring in HGSOC.

Other animal models such as hens or rats have also been suggested (Stewart, Querec et al. 2004).

Taken together, there are no satisfactory models to study the HGSOC so far. Creation and establishment of cell lines that reflect different histotypes and molecular subtypes of ovarian cancer, especially HGSOC will bring enormous benefits to the research field.

Molecular characteristics of HGSOG

96% of the high-grade serous ovarian cancers were reported to have mutations of the *TP53* tumour suppressor gene. Mutations of *BRCA1* and *BRCA2* were found in approximately 22% HGSOC and *KRAS*, *PTEN* or *BRAF* in less than 6% of all cases (Cancer Genome Atlas Research 2011).

Cytokeratin is a family of intermediate filament proteins in the intracytoplasmic cytoskeleton of epithelial tissues, which contains about 20 members. The expression of the subtype of cytokeratin by a certain epithelial cell is dependent on the type of epithelium, the status of differentiation and development of the cell. Cancer cells also present specific cytokeratin profiles. Cytokeratin 8, 18 and 19 are members of the keratin family and are often used together to identify cells of epithelial origin. In ovarian cancer they are widely expressed (Van Niekerk, Ramaekers et al. 1993).

Epithelial cell adhesion molecule (EpCAM) is a transmembrane protein which is involved in many cell pathways such as adhesion, migration, proliferation and differentiation. When epithelial cells express EpCAM, they can attach to the surrounding tissues, to each other or to the cell culture flask. When EpCAM is cleaved, as it is in

tumour cells, the molecule gains oncogenic potential. In breast cancer, it has been suggested that EpCAM expression is down-regulated during EMT, but then up-regulated once the tumour cells reach the remote organs (van der Gun, Melchers et al. 2010). EpCAM was reported to be highly expressed in most of the serous ovarian cancer and its overexpression was a prognostic marker for poor survival of the patients (Spizzo, Went et al. 2006).

CD44 is a transmembrane glycoprotein, which has different variants through alternative splicing. Different CD44 isoforms are expressed in a variety of cells including leukocytes, mesothelial cells and also tumour cells (Naor, Sionov et al. 1997). In ovarian cancer, CD44 is expressed in some mucinous and endometrioid epithelial tumours, but not in the serous ovarian cancer[4]. It has been demonstrated that CD44, its ligand hyaluronan and the interacting partner of hyaluronan versican are very important factors in the degradation of the extracellular membrane surrounding the ovarian tumours and in the facilitation of the peritoneal dissemination of tumour cells (Ween, Oehler et al. 2011). Furthermore, CD44 can be detected together with EpCAM on cancer initiating cells (Marhaba, Klingbeil et al. 2008).

Vimentin, like cytokeratins, is an intermediate filament protein, which is a specific marker for mesenchymal cells or mesenchymally derived cells. It detects mesenchymal cells in the stroma such as fibroblasts, smooth muscle cells and endothelial cells. It has also been reported that tumour cells undergoing EMT during metastasis have vimentin expression (Satelli and Li 2011).

Calretinin is a suitable immunohistochemical marker for mesothelial cells lining the peritoneal cavity, which are often found in ascites fluid (Doglioni, Dei Tos et al. 1996). Calretinin positive staining could also been observed on some locations in ovarian tumour tissues (Cao, Jones et al. 2001).

[4] http://www.proteinatlas.org/ENSG00000026508/cancer/tissue/ovarian+cancer [2013/11/15]

Research Question

General objective

The general aims of this project are to establish primary cell cultures or cell lines from ascites and/or tumour tissues of high grade serous ovarian cancer patients that can be used as models to study ovarian cancer, especially the resistance of ovarian cancer.

Special scientific and technical objectives

Establishment of primary cell cultures / cell lines

- To collect ascites and tumour tissues of patients with high grade serous ovarian cancer
- To isolated cells from clinical materials and set cell culture
- To optimize and standardize the culture conditions and procedures
- To obtain pure tumour cell cultures or cell lines

Analyses of the cell cultures / cell lines

- To observe and document the morphology of cells and their growth
- To extract DNA and RNA from cultured cells
- To prepare cytospin of cells
- To identify the *TP53* gene mutations in tumour tissues using a functional yeast assay
- To examine the cell cultures for their *TP53* mutation status by direct sequencing or droplet digital PCR
- To analyse the protein expression of the cultured cells with immunohistochemistry (IHC) or immunofluorescent (IF) staining
- To prepare sections from formalin-fixed paraffin embedded (FFPE) tumour tissue blocks
- To perform IHC in tumour tissues
- To expand and define tumour cell lines

Expected results:

Established and molecularly well characterised tumour cell lines which represent high grade serous ovarian cancer.

Materials and Methods

Materials

Patients with epithelial ovarian cancer were included in this study at The Department of Obstetrics and Gynaecology, Medical University of Vienna, from April 2012 to August 2013. Informed consents have been obtained from all patients and the study protocol was approved by the local ethics committees (EK Nr. 366/2003). Ascites samples were drained from the peritoneum of 24 patients before any primary treatment. From 22 of these patients, tumour tissues were also available. The volume of the ascites obtained from the patients in this project varied between 200ml and 5L, whereas only 50ml to 200ml were used for cell culture.

Histopathological examination was performed at The Department of Pathology at the Medical University of Vienna.

Methods

Cell culture

All steps were performed under sterile conditions in a working bench Biowizard Golden GL-130, Class II (KOJAIR, Vilppula, Finland).

Initiation of cell culture from ascites

50-200 ml ascites were centrifuged at 1500g at 4°C for 20min. The supernatant was filtered through a 0.22µm membrane filter "Standard MF-Millipore Membranes" (Merck Millipore, Darmstadt, Germany) and 5ml was aliquoted and stored at -20°C for further supplement of the culture medium. The cell pellet was re-suspended in 10ml PBS (Gibco Life Technologies, California, USA). The red blood cells were depleted by overlaying cell suspension onto 10ml Histopaque 1077 (Sigma-Aldrich, St. Louis, USA) and centrifuged at 1000g for 30min at RT. The milky interface-layer was collected with a pipette and the cells were washed twice by adding 50ml PBS and centrifuge at 220g for 10min at RT. The cells are cultivated in DMEM medium (Gibco Life Technologies, California, USA)

supplemented with 10% Fetal Bovine Serum (FBS) (Gibco Life Technologies, California, USA), 10.000 Units/ml Penicillin and 10.000µg/ml Streptomycin (PS) (Gibco Life Technologies, California, USA) and 10% ascites supernatant. Cells were counted and seeded in appropriate size of cell culture flasks such as Nunclon Treated Tissue Culture Flasks with Screw Cap and Straight-Neck (Thermo Scientific Nunc, Massachusetts, USA) or Falcon Cell -Culture Flasks (Corning, New York, USA) according to the cell number.

Initiation of cell culture from tumour tissue

Tumour tissue was kept on ice until placed into a petri dish (⌀ 5cm) with 4.8ml DMEM+PS medium and was mechanically cut into very small pieces. The tissue was further digested with 250-500µl of 1mg/ml collagenase (Sigma-Aldrich, St. Louis, USA) for 30-60 min at 37°C. The amount of the enzyme and the digesting time were decided according to the size and texture of the tissue sample. The digestion was stopped by adding 5ml medium with 10% FBS+PS. In most cases, clusters of cells were released from the tissue. Cells were washed by adding 50ml PBS and centrifuged at 220g for 10min at RT. The cells are cultivated in DMEM medium. Depending on the size of the cell clusters, the tissue sample was eventually filtered through a strainer with adequate pore size of 40µm. Cells were then counted and seeded in appropriate sized cell culture flasks according to the cell number.

Maintenance and development of cell culture

Cells were cultivated at 37°C and with 5% CO_2. Medium was changed twice a week or according to the growth of the cells. For freezing cells in liquid nitrogen, complete medium was supplemented with 10% DMSO (Sigma-Aldrich, St. Louis, USA).

In order to remove the undesirable fibroblasts from the primary culture, selective trypsinisation was performed. In detail, 0.05% Trypsin-EDTA (1x) (Gibco Life Technologies, California, USA) was applied onto cells in culture flasks. The detachment of the cells were observed under a microscope and trypsinisation was stopped by adding fresh complete medium as soon as most of the fibroblasts detached and the tumour cells were still attached to the cell culture flask.

When splitting the cells, they were rinsed once with PBS and trypsinised for about 2-8 min at 37°C. The trypsin was inactivated by adding complete medium and the cells were washed once with PBS before being set into culture again. The splitting ratio was determined according to the growth rate, usually at 1:1.5 or 1:2.

Authentication of cells by analysing short tandem repeats (STRs)

Short tandem repeats or microsatellites are repetitive sequences of 2-6 base pairs of DNA. These sequences are different in every individual and are unique fingerprints of each individual. Some of these sequences are used as molecular markers for identification of relatives or matching DNA. To avoid the contamination of cultured cells, we examined the repeats by PCR and fragment analyses. In detail, 7 markers were examined (Table 1). All antisense primers were Cy5 labelled. PCR was performed with 40 cycles of denaturation at 94°C for 30sec, annealing at 62°C for 30sec, and elongation at 72°C for 30sec, preceded with an initial denaturation step at 94°C for 10min and a final elongation step at 72°C for 10min. PCR products were resolved on an acrylamide gel (ReproGel High Resolution, GE Healthcare, Uppsala, Sweden) on an ALF fragment analyser (GE Healthcare).

Table 1. Markers and their primer sequence used for STR analyses.

Marker		Primer sequence
TPOX	Sense	5'-GCACAGAACAGGCACTTAGG-3'
	Antisense	5'-CGCTCAAACGTGAGGTTG-3'
vWA	Sense	5'-GCCCTAGTGGATGATAAGAATAATCAGTATGTG-3'
	Antisense	5'-GGACAGATGATAAATACATAGGATGGATGG-3'
CSF18O	Sense	5'-CCGGAGGTAAAGGTGTCTTAAAGT-3'
	Antisense	5'-ATTTCCTGTGTCAGACCCTGTT-3'
D16S539	Sense	5'-GGGGGTCTAAGAGCTTGTAAAAAG-3'
	Antisense	5'-GTTTGTGTGTGCATCTGTAAGCATGTATC-3'
D7S820	Sense	5'-ATGTTGGTCAGGCTGACTATG-3'
	Antisense	5'-GATTCCACATTTATCCTCATTGAC-3'
D13S317	Sense	5'-ATTACAGAAGTCTGGGATGTGGAGGA-3'
	Antisense	5'-GGCAGCCCAAAAAGACAGA-3'
D5S818	Sense	5'-GGTGATTTTCCTCTTTGGTATCC-3'
	Antisense	5'-AGCCACAGTTTACAACATTTGTATCT-3'

Morphologic examination of cells

Microscopic examination

Cells were examined under a microscope Olympus BX50 (Olympus, Tokyo, Japan) and pictures were taken with a "Soft Imaging System CC12" (Olympus, California, Japan).

Preparation of cytospins

Cells were counted and suspended at no more than $5x10^5$ cells/ml in PBS. 500µl suspension was loaded in each cytospin-cuvette on a poly-L-lysine coated glass slide (Thermo Scientific, Massachusetts, USA) and spun at 160g for 5min at RT. Supernatant was removed and slides were dried overnight and stored at -20°C until staining.

Preparation of chamber slides

8-well chamber slides, µ- Slide 8-well (ibidi, Martinsried, Germany), were coated with 1ml of 1mg/ml Poly-D-Lysine in H_2O suspension and incubated for 1h at RT. The suspension was removed and the chamber slides were dried overnight and stored at -20°C until further use.

The cells were suspended in a concentration less than $5x10^4$ cells/ml in medium. 300µl were added in each chamber. The growth and the attachment of the cells should be observed twice a day. Usually after 2 to 4 days, the medium was removed and the slides were dried overnight and stored at -20°C until staining.

Hematoxylin and Eosin (HE) staining

Slides were first placed in Xylol for 10min to remove the paraffin. To rehydrate tissue, slides were placed in descending ethanol series (2x 100%, 5min; 1x 96%, 5min; 1x 80%, 5min; 2x 70%, 5min). They were rinsed in distilled water for 5min and then placed for 5min in Papanicolaou's solution 1a Harris' hematoxylin solution (Merck Millipore, Darmstadt, Germany) for nucleus staining, followed by a thorough rinse under tap water for at least 10min. Counterstaining is performed by putting slides for 1min in a EosinY disodium salt solution (Sigma-Aldrich, St. Louis, USA) and then rinsed shortly in water. The slides were then proceeded in ascending ethanol series (1x 70%, 1x 80%, 1x 96%, 2 x 100%) and finally in Xylol for 5-10min. They were then covered with Pertex mounting media (Leica, Solms, Germany).

Genomic analysis

Isolation of DNA and RNA from tumour tissue

Fresh tumour tissue of about 30-50mg was snap frozen in liquid nitrogen and immediately placed at -80°C until nucleic acid isolation. The tumour tissue was homogenized by a Mikro-Dismembrator U (B.Braun, Maria Enzersdorf, Austria). A AllPrep DNA/RNA Mini Kit (Qiagen, Hilden, Germany) was used to isolate DNA and RNA. The concentrations were measured by BioPhotometer (Eppendorf, Hamburg, Germany).

Determination of *TP53* mutations in tumour tissue

TP53 gene mutations in tumour tissues were first identified using a p53 functional assay of separated alleles in yeast (FASAY). The protocol was developed by the functional yeast-based assay originally described by Flaman et al. (Flaman, Frebourg et al. 1995). In principal, mutant *TP53* losing its transcriptional activation function, can be transformed to a recipient yeast strain ylG397, which is defective in adenine synthesis. This defect is caused by a mutation in the ADE2 gene. However, this strain contains another copy of ADE2, whose open reading frame is regulated by *TP53* gene. Wild type *TP53* can initiate the transcription and the yeast will grow normally and form white colonies. Mutant *TP53* fails to initiate the transcription of ADE2 and the yeast therefore cannot grow on a normal agar plate. However, they are able to grow in low-adenine plate and form red colonies. In this way, mRNAs containing *TP53* mutations can be identified in the red colonies. Briefly, mRNA was isolated from total RNA with MPG mRNA Purification Kit (PureBiotech, USA) and transcribed into cDNA by reverse transcription with Omniscript RT Kit (Qiagen, Hilden, Germany). PCR was performed with self-designed primers (P3 and P4, see Index B) for 40 cycles of 30sec at 94°C, 60sec at 65°C and 80sec at 78°C. PCR products were transformed into the yeast strain yIG397, which was subsequently cultivated on agar plates (for composition see index B). Linearised pRDI-22 and sonicated salmon sperm DNA were used as vector and carrier DNA, respectively. Yeast grew for 2 days at 37°C.

Samples were defined as mutated when red colonies were present and mutation was identified and confirmed by direct sequencing. Red colonies were suspended in 20µl

water of which 2µl was used for amplification of *TP53* sequence with primers C1 and C2 (see Index B). PCR was performed for 40 cycles of 30sec at 94°C, 60sec at 65°C and 80sec at 78°C. PCR products were purified with the GFX PCR DNA and Gel Band Purification Kit (Amersham Bioscience, Uppsala, Sweden).

Direct Sequencing after Sanger

Direct sequencing is also called Sanger sequencing. It is often used to detect nucleotide base. This method can only detect up to 20% sensitivity. Although *TP53* consists of the coding regions from exon 2 to exon 11 and additional intron regions which can be mutated, we only sequenced exon 4 to exon 10 because mutations outside of this part of the gene occur very rarely.[5]

Determination of KRAS mutations in tumour by reverse oligonucleotide hybridisation method – Strip assay

Hot spot mutations in KRAS (Codon 12 and Codon 13) were detected with a reverse oligonucleotide hybridisation method by a collaborator ViennaLab Diagnostics (Vienna, Austria) and confirmed by direct sequencing (Zimmermann, Voss et al. 1988).

The principle of this method is a PCR followed by hybridisation for the mutated and the wild type sequences to a membrane, on which the specific oligonucleotides were bund to. Even when the target sequences appear at 2% of the total samples, they could be detected using this very sensitive method (Tag, Oberkanins et al. 2008).

Droplet digital PCR

Droplet digital PCR (ddPCR) is based on the detection of each DNA copy and has the highest detection sensitivity. Through the repartition of reagents and samples into 20,000 droplets and the subsequent PCR, the target nucleic acids can be quantified by counting the droplets with positive fluorescent signal and negative ones in the ddPCR

[5] http://p53.free.fr/Database/p53_database_distr.html [2013/12/05]

system (Bio-Rad, California, USA). The digital readout provides the absolute copy numbers of the target DNA (Figure 3).

In details, DNA sequence of interest was labelled with either fluorescein amidite dye (FAM), which gives a blue fluorescent signal or VIC dye, which gives a green fluorescent signal. In our experiment the mutant type was labelled with FAM, whereas the wild type was labelled with VIC. A PCR was performed in the ddPCR Droplet Generation Oil (Bio-Rad) generated in the QX100 Droplet Generator (Bio-Rad) and analysed by the QX100 Droplet Reader (Bio-Rad) and the software QuantaSoft (Bio-Rad) (Hindson, Ness et al. 2011).

Five ddPCR systems were established. Primers and probes were shown in table 2. PCR was performed with 40 cycles of denaturing at 94°C for 30sec and annealing/elongation at 60°C for 1min, which was proceeded with a 10min initial denaturation step at 95°C and followed by a final elongation at 98°C for 10min.

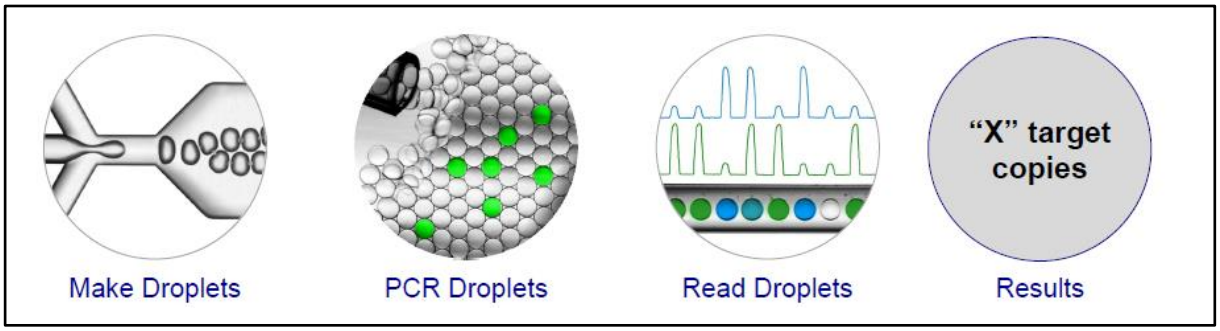

Figure 3. Workflow of ddPCR. (http://www.bio-rad.com/de-at/applications-technologies/droplet-digital-pcr-ddpcr-technology#2)

Table 2. Primers and probes used for the ddPCR.

Mutation	Codon	Primers and probes
TAC(Tyr)-TGC(Cys)	234	Sense primer: 5'-GGCCTGTGTTATCTCCTAGGTTGGC-3' Antisense primer: 5'-CTTCCAGTGTGATGATGGTGAGGATG-3' WT probe: 5'-FAM-CACCATCCACTGCAACTACA-MGB-3' Mutant probe: 5'-VIC-ACCACCATCCACTACAACTACA-MGB-3'
GGC(Gly)-AGC(Ser)	244	Sense primer: 5'-CTAGGTTGGCTCTGACTGTACCAC-3' Antisense primer: 5'- GCTCCTGACCTGGAGTCTTCC-3' WT probe: 5'-FAM-CTGCATGAGCGGCAT- MGB-3' Mutant probe: 5'-VIC-CTGCATGGGCGGC- MGB-3'
CGG(Arg)-CAG(Gln)	248	Sense primer: 5'-TGTAACAGTTCCTGCATGGGC-3' Antisense primer: 5'-ACAGCAGGCCAGTGTGCA-3' WT probe: 5'-FAM- CATGAACCAGAGGCC-MGB-3' Mutant probe: 5'-VIC-CATGAACCGGAGGCC-MGB-3'
CGT(Arg)-CAT(His)	273	Sense primer: 5'-CTGAGTAGTGGTAATCTACTGGGACG-3' Antisense primer: 5'-CGGAGATTCTCTTCCTCTGTGC-3' WT probe: 5'-FAM-GAGGTGCATGTTTG-MGB-3' Mutant probe: 5'-VIC-GAGGTGCGTGTTTG-MGB-3'
del 3bp-ACG	170	Sense primer: 5'-CGCCATGGCCATCTACAAG-3' Antisense primer: 5'-GATAGCGATGGTGAGCAGCTG-3' WT probe: 5'-FAM-GTTGTGAGGCACTGCC-MGB-3' Mutant probe: 5'-VIC-GTTGTGAGGCGCTGCC-MGB-3'

Protein expression

Immunohistochemistry (IHC) is the process of detecting antigens in cells by the antibody antigen reaction. This method is an effective way to distinguish the types of cells because each type of cell has its specific antigen expression pattern. Primary and secondary antibodies used are listed in table 3.

Immunofluorescence staining

For fixation, the slide was incubated with 4% Formaldehyde in PBS (Sigma-Aldrich, St. Louis, USA) for 10min at RT, washed three times with PBS and further incubated with 0.5% X Triton™ X-100 (Sigma-Aldrich, St. Louis, USA) in PBS for 10min at RT for permeabilisation. Cells were then washed three times with PBS. Cells were then blocked by incubating slides with 2 drops of Ultra V Block (Agilent Technologies, Vienna, Austria) for 7min at RT to avoid unspecific binding, leading to background, and then washed with distilled water. Then slides were incubated with 200µl primary antibody (see table 3) diluted with Dako Antibody-diluent (Agilent Technologies, Vienna, Austria) overnight at 4°C in chamber. For each staining, a negative control with Dako IgG

antibody isotope control (Agilent Technologies, Vienna, Austria) or Antibody-diluent alone was realised. Then slides were washed three times with PBS-Tween (rinse first, separate washing). Secondary antibody are labelled with Alexa Fluor Dyes (Life Technologies, California, USA) (see table 3) was prepared in 6% Bovine Serum Albumin (PAA, Pasching, Austria) in PBS in absolute darkness. Secondary antibody was then incubated for 60min at RT in darkness. Cells were then washed three times with PBS. For nuclear counterstain slides were incubated for 5min at RT with DAPI (10000µg/ml, 1:10000 in PBS) (Life Technologies, California, USA) and then washed with PBS. One drop of Fluoromount-G (Southern Biotech, Birmingham, USA) was applied on coverslip and cover slide was placed on slide and dried overnight in the dark.

Table 3. Overview of primary and secondary antibodies.

Antibody		Clone	Target	Dilution
Primary Antibody	cytokeratin 8, 18 and 19, IgG1 mouse	A45-B/B3	Epithelial cells and tumour cells	1:100
	Vimentin, IgG2a mouse	2D1	Fibroblasts, some B- and T-lymphocytes and mammary carcinoma cell lines	1:500
	CD44, IgG1 mouse	8E2F3	Various cells amongst others immune and tumour cells	1:4000
	Calretinin, IgG rabbit	DC8	Normal Pleura lining/ malignant esothelioma	1:100
	EpCAM, IgG rabbit	E144	Epithelial cells and adenocarcinoma cells	1:300
Secondary Antibody	IgG1 mouse 568		IgG1 mouse primary antibodies	1:1000
	IgG2a mouse 488		IgG2a mouse primary antibodies	
	IgG mouse 568		IgG mouse primary antibodies	
	IgG rabbit 488		IgG rabbit primary antibodies	

Immunohistochemical staining

For tumour tissue staining, paraffin embedded sections of samples were stained by immunohistochemistry prior to additional Hematoxylin and Eosin stains.

To remove paraffin, slides with tissue slices were incubated for 1 hour at 58°C and then placed into Xylol (Fisher Scientific, Loughborough, UK) (2x for 5min). To rehydrate tissue, slides were placed into descending ethanol series (2 x 100%, 5min; 1x96%, 5min; 1x80%, 5min; 2x70%, 5min). Slides were rinsed in distilled water for 5min. Antigen retrieval was performed by heating up slides in citrate buffer with pH 6.0 (Merck Millipore, Darmstadt, Germany) in the microwave at 850W for 2.5min and then at 160W for 11min. They were cooled down slowly to RT. To block endogenous peroxidase activity, slides were placed in 3% Hydrogen peroxidase 30% (Merck Millipore, Darmstadt, Germany) in methanol (Fisher Scientific, Loughborough, UK) for 10min. Samples were again washed twice in distilled water for 2min. To block and prevent unspecific binding of the primary antibody, tissue samples were covered by Dako Ultra V Block (Agilent Technologies, Vienna, Austria) for 7min. Slides were washed twice in PBS-Tween for 3min. Then the primary antibody (see table 3) was diluted with Dako Antibody-diluent (Agilent Technologies, Vienna, Austria) and incubated over night at 4°C. For the negative control, Dako Negative Control Ig mouse cocktail (Agilent Technologies, Vienna, Austria) or Antibody-diluent alone, was used. After incubation overnight, samples were first rinsed and then separately washed twice with PBS-Tween for 3min. Then the staining kit LSAB+ System – HRP Code K0690 from Dako (Agilent Technologies, Vienna, Austria) was used in a three step procedure: first step Biotinylated link was added for 15min, then Streptavidin-HRP for 15min and finally the DAB+ (substrate buffer + chromogen) for 1-2min depending on the developing time of the reaction. Once brown staining is visible under microscope, reaction is stopped by washing slides twice in distilled water for 3min. To stain nucleus, hematoxylin staining with Hematoxylin solution modified acc. to Gill III (Merck Millipore, Darmstadt, Germany) was performed for 10sec and then immediately washed under flowing water for at least 10min. Finally ascending ethanol series (1x70%, 5min; 1x80%, 5min; 1x96%, 5min; 2 x 100%, 5min) and Xylol incubation (2x for 5min) was done following by the covering of the slides with Pertex mounting media (Leica, Solms, Germany).

Results

24 patients with EOC have been included in the study from The Department of Obstetrics and Gynaecology, at the Medical University of Vienna, from April 2012 to August 2013. 50ml to 200ml of ascites from all patients were processed to cell culture. Most of the patients had their corresponding tumour tissue also available for cell culture. However, as the success rate of establishing tumour tissue into primary culture is very low and the workload relatively high, we decided to continue the approach with only ascites. Therefore, only one cell line was established from tumour tissue.

Clinical and histopathological characteristics of the patients

Of the 24 patients, 23 were diagnosed with epithelial ovarian cancer and 1 patient with fallopian tube cancer. The age of the patients at diagnosis ranged from 26 to 75 with a median age of 50. The characteristics of the patients are summarised in Table 4. Among the 22 patients with serous ovarian cancer, one was identified to also have focal endometrioid tumours and another with borderline tumour parts. The patient with fallopian tube carcinoma was not classified regarding the histological type.

The majority of the patients (20) have FIGO stage III, which corresponds to a cancer growth in one or both ovaries with the spread of tumours to the lining of the abdomen but without distant metastasis. One patient had FIGO I, one had FIGO II and two patients had distant metastasis and therefore FIGO IV.

Most tumours had high-grade differentiation (19 with G3 and two with G2), only two patients had high differentiated (G1) ovarian cancer.

13 of the 21 patients which were operated on, were fully debulked with no macroscopically visible residual tumours and 8 had residual tumours after the surgery. Three patients had no surgery.

Table 4. Clinical and histopathological characteristics of 24 epithelial ovarian cancer patients.

Histological type	**Number**
Serous	22
Endometrioid	1
Mucinous	0
Clear cell	0
Not classified	1
FIGO	
I	1
II	1
III	20
IV	2
Grading	
G1	3
G2	2
G3	19
***TP53* mutation status**	
Mutated	18
Non mutated	6
***KRAS* mutation status**	
Mutated	3
Non mutated	21
Rest tumour	
Yes	8
No	13
No debulking	3
Age	
≥50	16
<50	8

Establishment of the cell lines

From both ascites and tumour tissues obtained from the 24 patients, cell culture was set up. Two types of cells could be cultivated, which we describe as polygonal cells and tumour cells. In total, 13 primary cell cultures / cell lines were generated from materials of 11 patients, 10 of which harboured *TP53* mutation and one *KRAS* mutation in the corresponding tumour tissues and in two patients, both types of cell lines could be established (table 5). One tumour cell line was derived from tumour tissue of one patient, while all other cultures were generated from ascites.

Cell cultures from materials of other patients were also set up. In most cases, tumour cells could be observed with different proportions in the cell mixture. Some of them became adherent to the culture flasks and grew for a certain period of time, then finally died. Others died as floating clusters of cells, leaving amounts of cell debris in the culture medium. A number of cultures were contaminated with bacteria or fungi.

Table 5. Overview of all established primary cell cultures / cell lines.

Cell line	Morphology	Highest passage number	Histological type	FIGO	G	*TP53* in tissue	Type of mutation	*TP5* % Ascites ddPCR	*TP5* % Ascites FASAY
12245	polygonal	↯ 8	serous	IIIC	3	TAC(Tyr)-TGC(Cys) Codon 234	missense	46%	99%
12370	cluster	> 39	serous	IIIC	3	CAT(His)-CCT(Pro) Codon 193	missense	n. a.	90%
12636	cluster	> 6	serous	IIIC	3	GGC(Gly)-AGC(Ser) Codon 244	missense	98%	100%
13070	polygonal	↯ 12	serous	IIC	3	CGG(Arg)-CAG(Gln) Codon 248	missense	4%	4%
13088	polygonal	↯ 18	serous	IIIB	3	CGT(Arg)-CAT(His) Codon 273	missense	3%	0%
8587 (tissue)	adherent	> 30			3				
13363	adherent	> 21	serous	IV	3	del 3bp-ACG Codon 170	missense	25%	70%
13646	polygonal	↯ 6	serous	IIIB	3	wild type	*KRAS* mutation	n. a.	0%
13699	cluster	> 6	serous	IIIC	3	CGT del C Codon 333	frameshift	n. a.	10%
13781	cluster	> 4	serous	IV	3	GTG(Val)-ATG(Met) Codon 272	missense	n. a.	75%
13848	polygonal	↯ 6	serous	IIIC	3	GAG(Glu)-AMB Codon 224	nonsense	n. a.	0%
13914-1	adherent	> 25	serous with focal endometrioid part	IIIC	3	GAG-del 10-AG-CTG Codon 339-343	frameshift	n. a.	15%
13914-2	polygonal	↯ 10							

Note: ↯ indicates that the cells normally died at around this passage; n.a.: samples were not analysed.

Morphology and growth

Two types of cells could be observed in primary culture.

6 primary cultures presented with uniform polygonal form, had relative large sizes and relative small nuclei. The nucleus/cytoplasm ratio was relatively lower (figure 4A). They grew in monolayer and proliferated very rapidly with a doubling time of around 1 day from the beginning until around the 5th passage (figure 4B). They adhered to the plastic culture flask very loosely and could be detached easily with trypsinisation. From a certain passage onwards, the cells ceased to divide. They became enlarged, lost contact with other cells and reached a “replicative senescence” (figure 4C). Most of them died between the 6^{th} and the 12^{th} passages.

In the other 7 cultures, cells had a polymorphic appearance. They showed irregular sizes and shapes, had a large nucleus and a relatively higher nucleus/cytoplasma ratio (figure 4D). These cells adhered to the plastic surface (3 lines) or grew mainly as clusters in the medium (4 lines). The adherent cells often formed an island-like structure surrounded

by fibroblasts (figure 4E) at the beginning of culture which mimicked the tumour structure *in vivo*. The fibroblasts were depleted by selective trypsinisation, forming pure tumour cell culture (the evidence of molecular analysis will be given in the later chapters). Two adherent cell lines are currently in the 30th and 25th passage of culture with a doubling time of around 3-4 days, another is in the 22th passage of culture with a doubling time of about 7-10 days. The clusters could contain a couple of cells up to several hundred of cells (figure 4F). Sometimes they switched to adherent growth. A great number of cells died during the proliferation, creating a lot of debris in the culture. These cell lines had different proliferation rates. Currently, we have one line in the 40th passage of culture and three between the 4th and 6th passage.

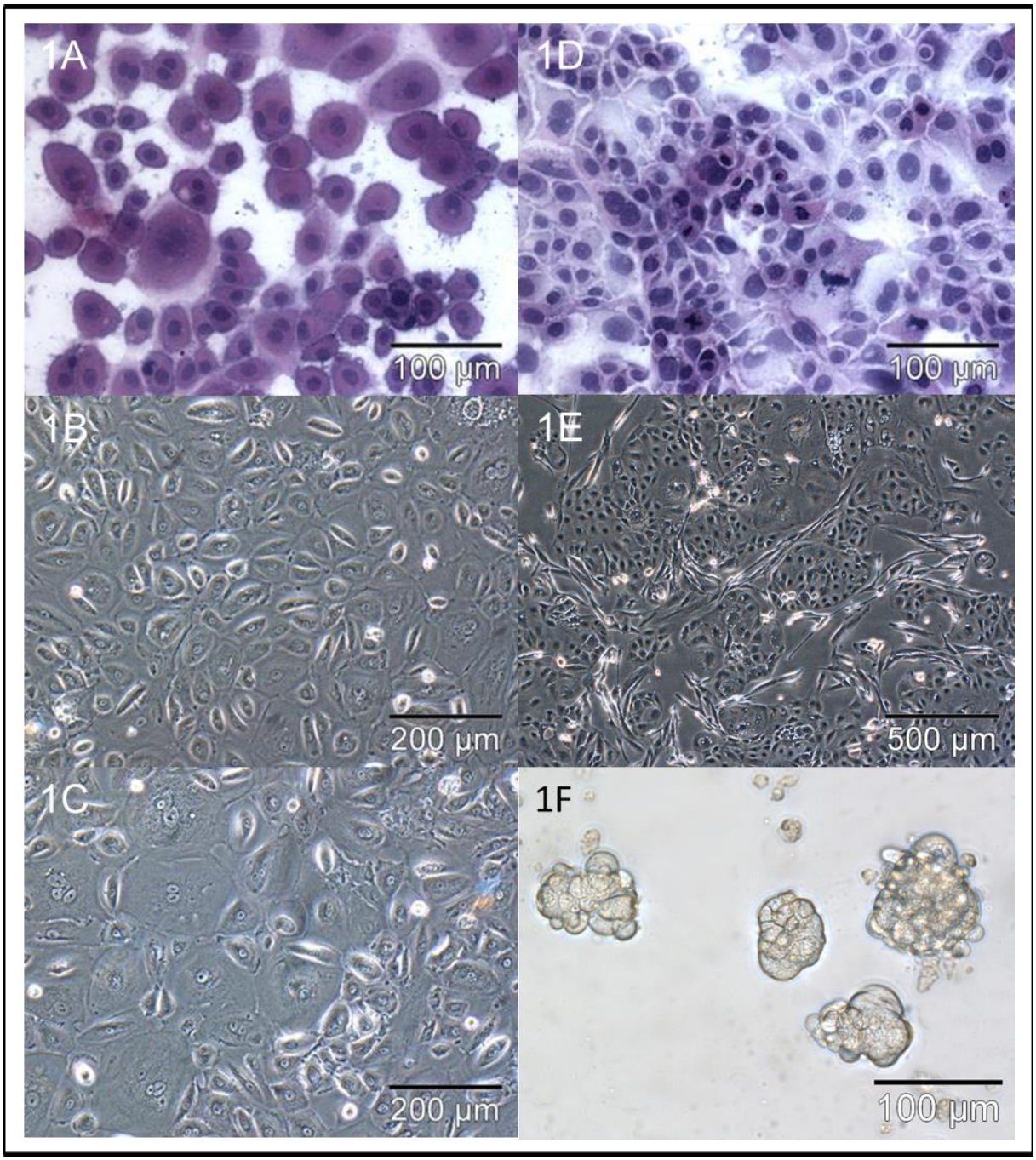

Figure 4. Cell morphology of primary cell cultures / cell lines. 4A: polygonal cells growing on a chamber slide and with HE staining; 4B: polygonal cells at 8th passage of culture; 4C: polygonal cells at 12th passage of culture; 4D: tumour cells growing in chamber slide and with HE staining; 4E: tumour cells constructing island-like structures; 4F: cluster of tumour cells.

Molecular characteristics of the cell lines

Authentication of the cells

To ensure that the cells originated from the original tissues or ascites of a certain patient and were not contaminated with some rapidly growing cells, we carried out the authentication examination of the cells regularly by determining seven short tandem repeat (STR) markers in the DNA of the cells and comparing the results with the corresponding tumour tissues and blood samples of the patients.

All primary cultures and/or cell lines were confirmed to originate from the same patients. An example of the result was shown in figure 5.

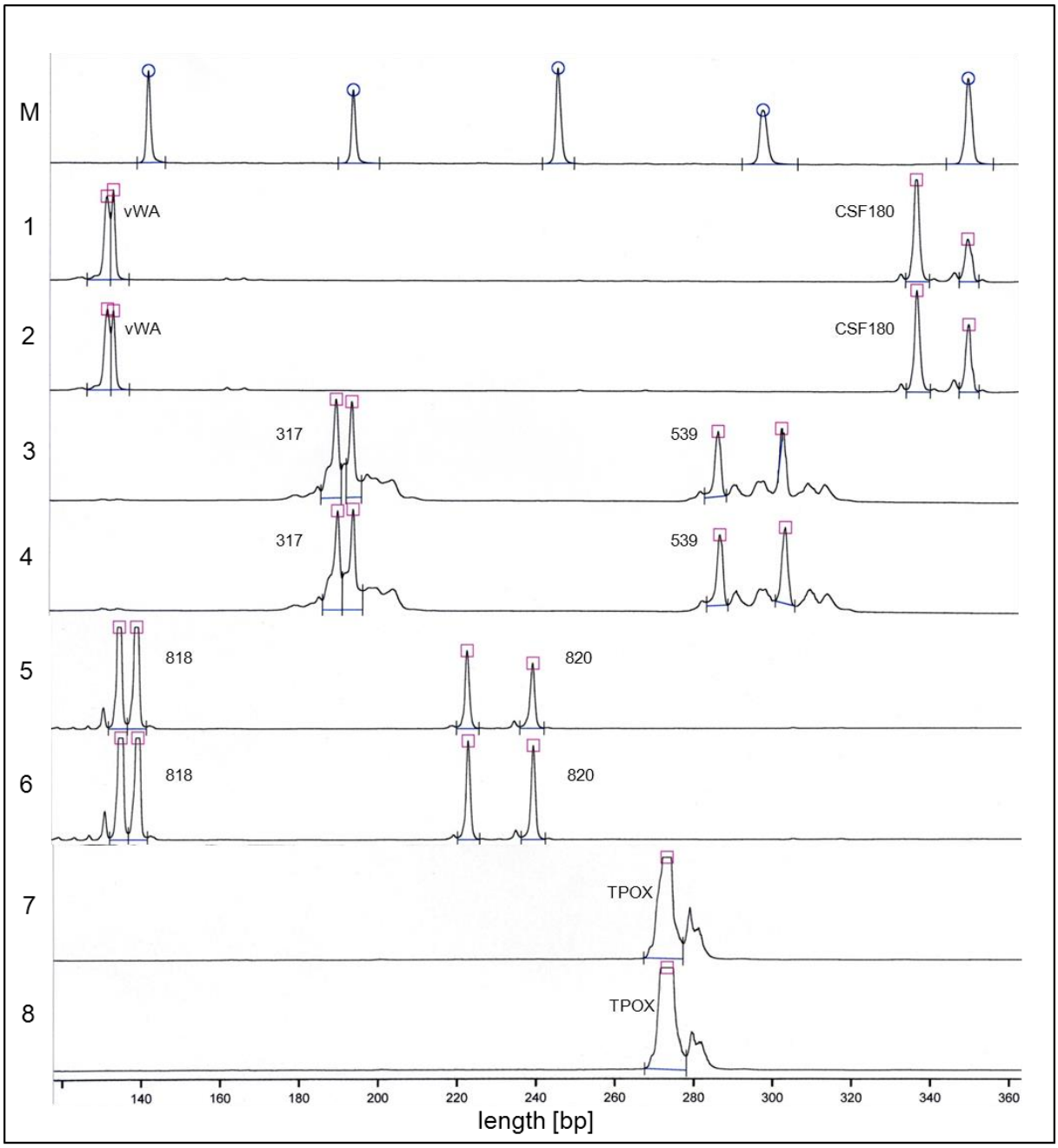

Figure 5. One example of the determination of the short tandem repeat (STR) by ALF express device. Lane M: Marker containing DNAs with defined lengths; Lanes 1, 3, 5, 7: cell culture 13088 at passage 0 with STR markers vWA, CSF180, 317, 539, 818, 820 and TPOX; Lanes 2, 4, 6, 8: the same culture at passage 4.

Technical results

The functional yeast assay

Figure 6 shows examples of the result of the functional yeast assay. The assay was always performed with a positive and a negative control sample (figure 6A, 6C). Wild type *TP53* DNA results in white colonies and mutant DNA in red colonies.

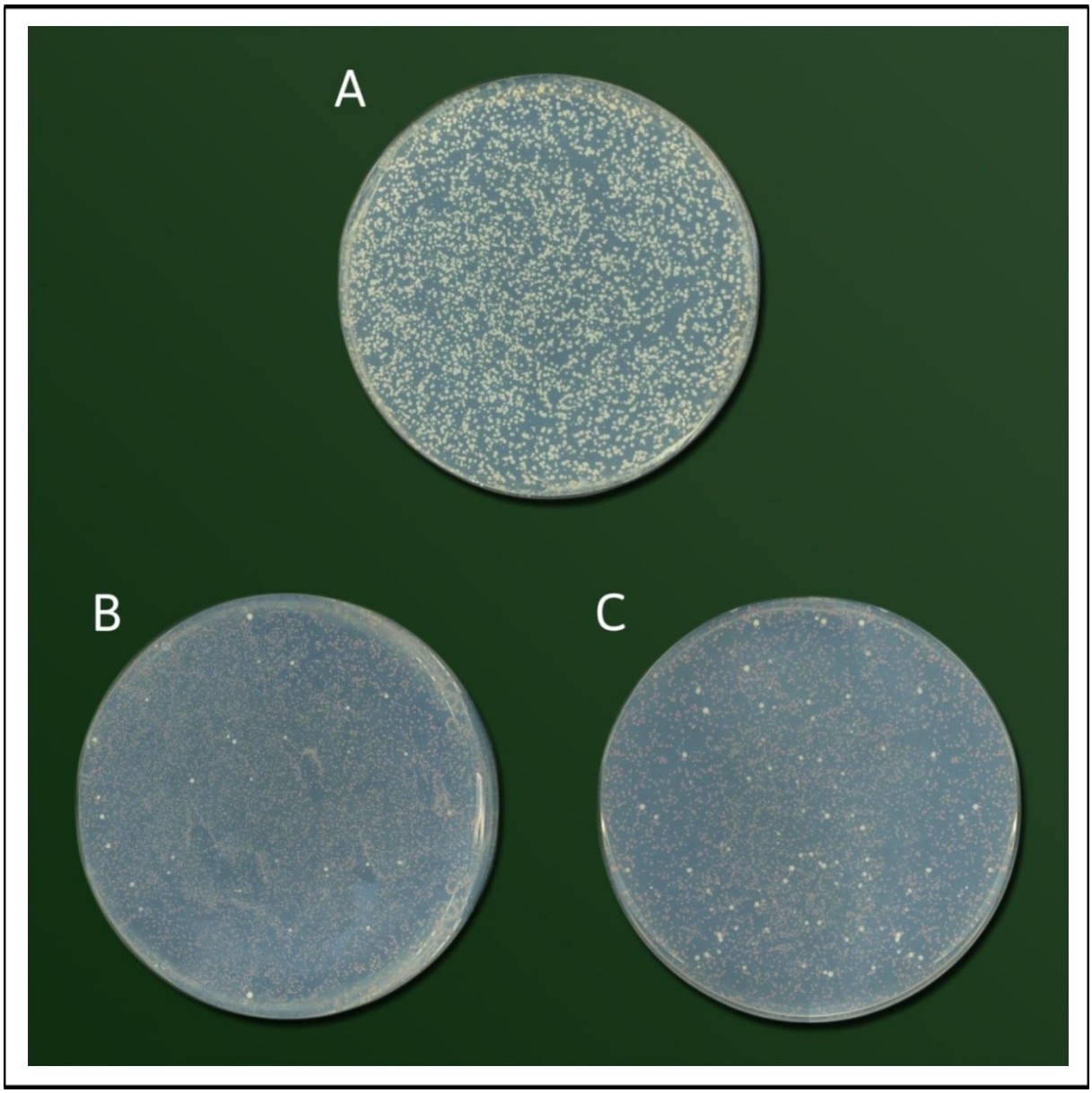

Figure 6. Example of FASAY results from cells in primary culture 12636 at passage 3. 5A: wild type control; 5B: the sample; and 5C: mutant control.

Direct sequencing

The colonies were picked up and the sequence was amplified and processed for direct sequencing. *TP53* mutations were determined by comparing the sequence with a standard *TP53* wild type sequence. Figure 7 illustrates a sequencing result from primary culture of sample number 13070. A *TP53* mutation CGG to CAG causing an amino acid change of arginine to glutamine at the codon 248 was identified in the tumour tissue. Cells from the ascites of the patients showed 4% red colonies. Sequencing of these colonies confirmed that they indeed had the same *TP53* mutation as in the tumour.

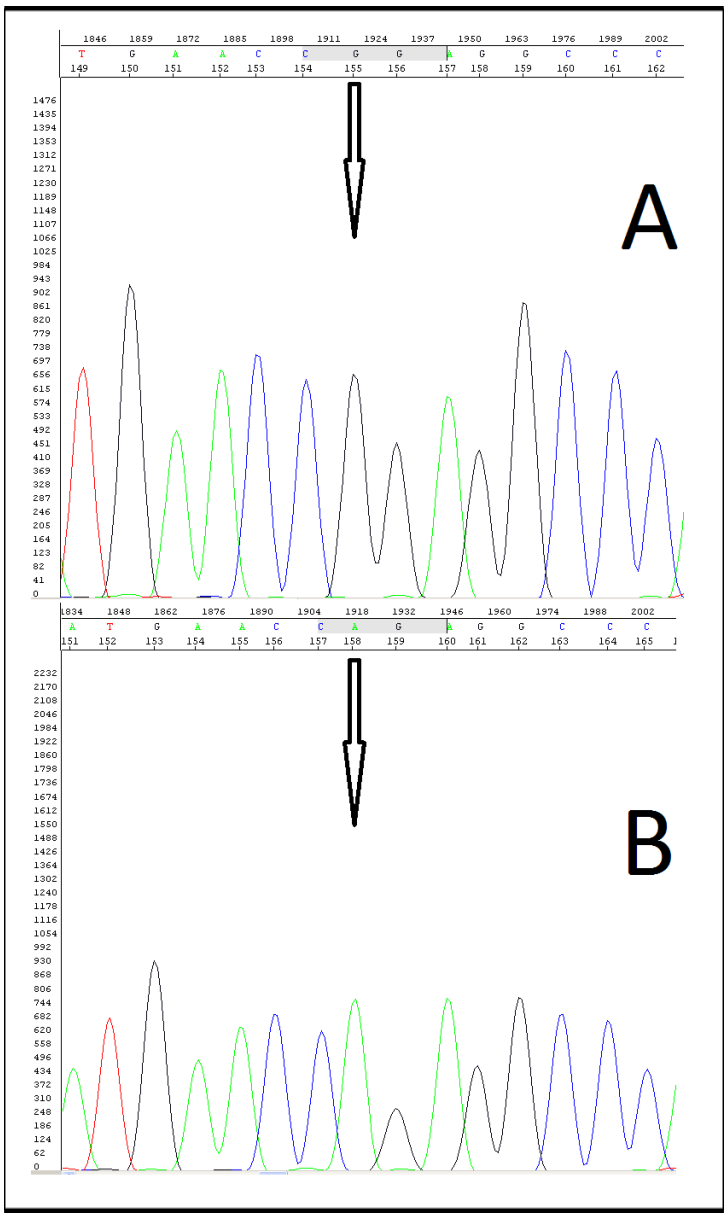

Figure 7. Sequencing results showing a homozygote point mutation of *TP53* at codon 248; A: a fragment of wild type sequence of *TP53* gene. B: a G to A point mutation at codon 248 causing a change of amino acid of arginine to glutamine in sample number 13070.

FASAY is a very useful tool to identify *TP53* mutation in samples with unknown *TP53* mutation status. Direct sequencing helps us to determine the *TP53* mutation. FASAY, however, is based on mRNA, which is transcribed to cDNA. In different cells, the number of the mRNA transcripts varies a lot. Thus, the proportion of the red colonies in the FASAY does not represent the proportion of the mutant cells, from which the RNA was extracted. Direct sequencing of DNA is easy to perform and can give us a quick result that enables us to roughly estimate the purity of tumour cells in culture (Figure 8).

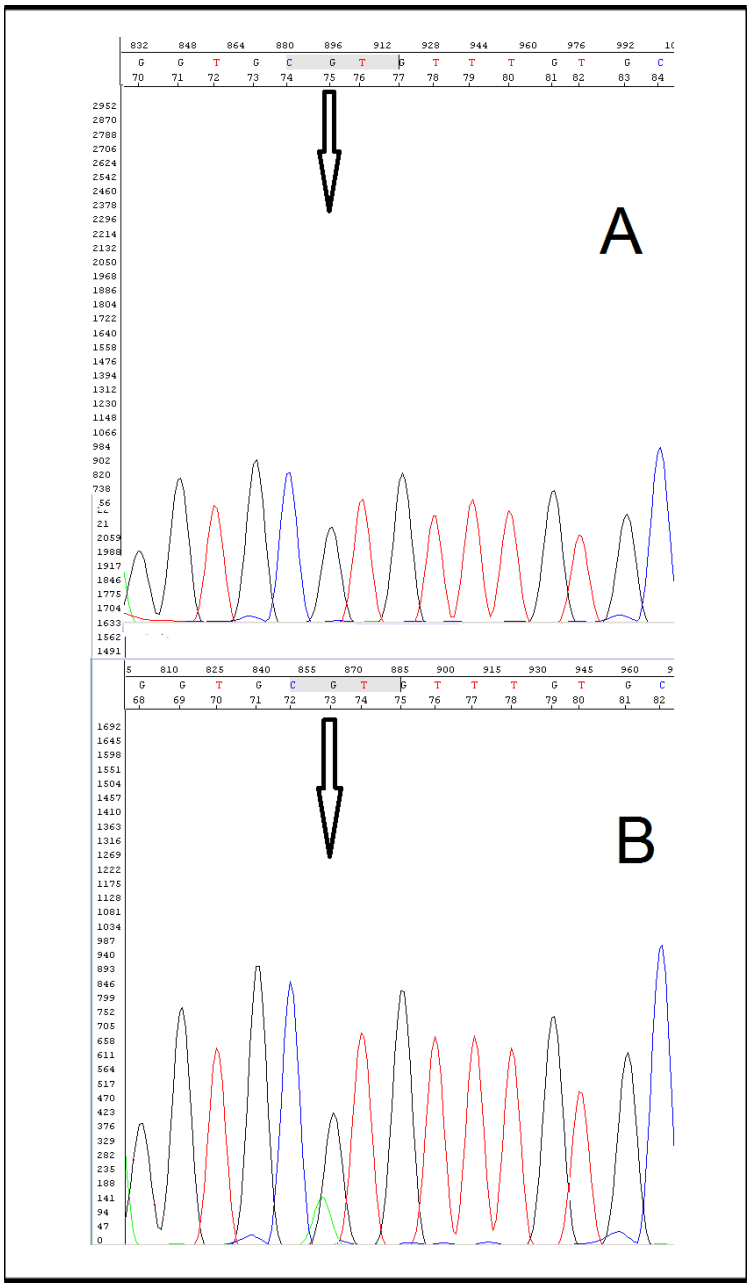

Figure 8. Sequencing results showing a heterozygote point mutation of *TP53* at codon 273: A: a fragment of wild type sequence of *TP53* gene. B: a G to A point mutation at codon 273 causing a change of amino acid of arginine to histidine, the sample consists of both wild type and mutant cells.

Droplet digital PCR (ddPCR)

The systems were optimized so that mutant probes and wild-type probes specifically bind to their complementary DNA. Cell lines with known *TP53* mutation and wild type samples were used to evaluate the sensitivity and specificity of the assay.

By comparing the samples with mutant and wild type standards, the false positive and false negative events introduced by autofluorescence or other reasons could be excluded. Figure 9 presents an example of the results of one sample with an overwhelming number of mutant copies of tumour cells.

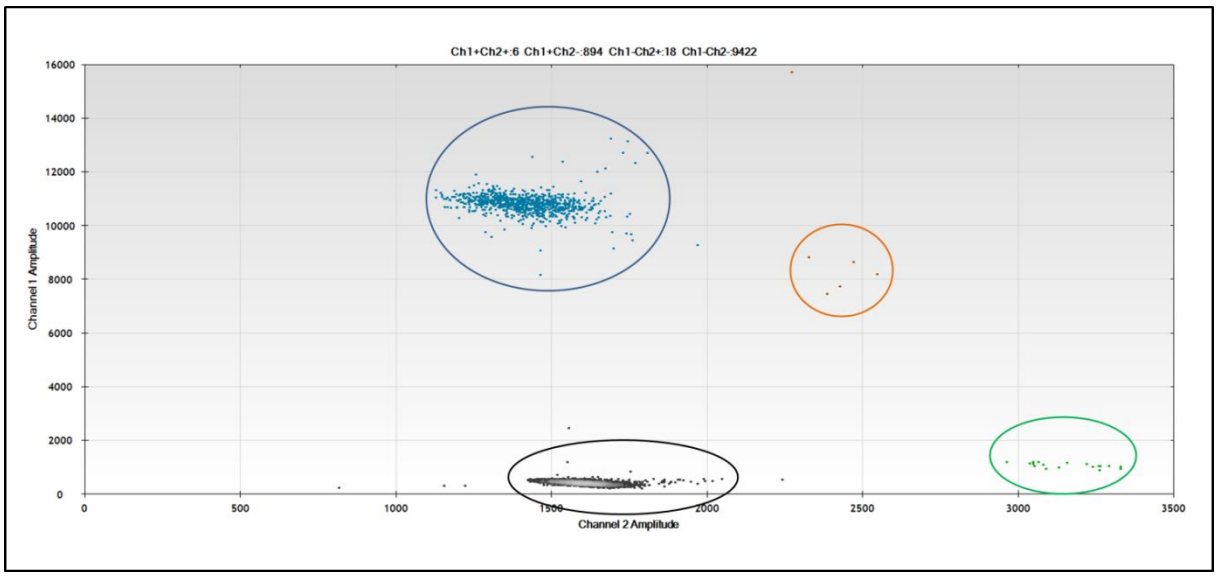

Figure 9. Result of ddPCR from cell line 12636 at passage 3. Blue area: mutant droplets (events: 894 events); green area: wild type droplets (18 events); orange area: double positive (6 events); black area: empty droplets with autofluorescence with low intensity. The estimated proportion of tumour cells in the culture is: (894+6)/(18+894+6+6)=96.77%.

Reverse oligonucleotide hybridisation to detect mutations in the *KRAS* gene

In one of the primary cultures set from ascites of a HGSOC patient, no *TP53* mutation could be identified. Therefore, we examined the common *KRAS-BRAF* mutations. The KRAS-BRAF StripAssay (ViennaLab Diagnostics GmbH, Vienna, Austria) is based on reverse-hybridisation of biotinylated PCR products to a test strip, onto which allele-specific oligonucleotides were immobilized (Kriegshauser, Auner et al. 2011). It can detect eight mutations at codon 12 and two mutations at codon 13 of the *KRAS* gene and one mutation at *BRAF* gene (Figure 10). The DNA samples were processed in the

ViennaLab in frame of a cooperation project. Mutations in the samples could be read directly on the strips. The cells from sample number 13646 without *TP53* mutation was confirmed to have a G to T mutation at codon 13 of the *KRAS* gene causing an amino change from glycine to cysteine (Figure 10).

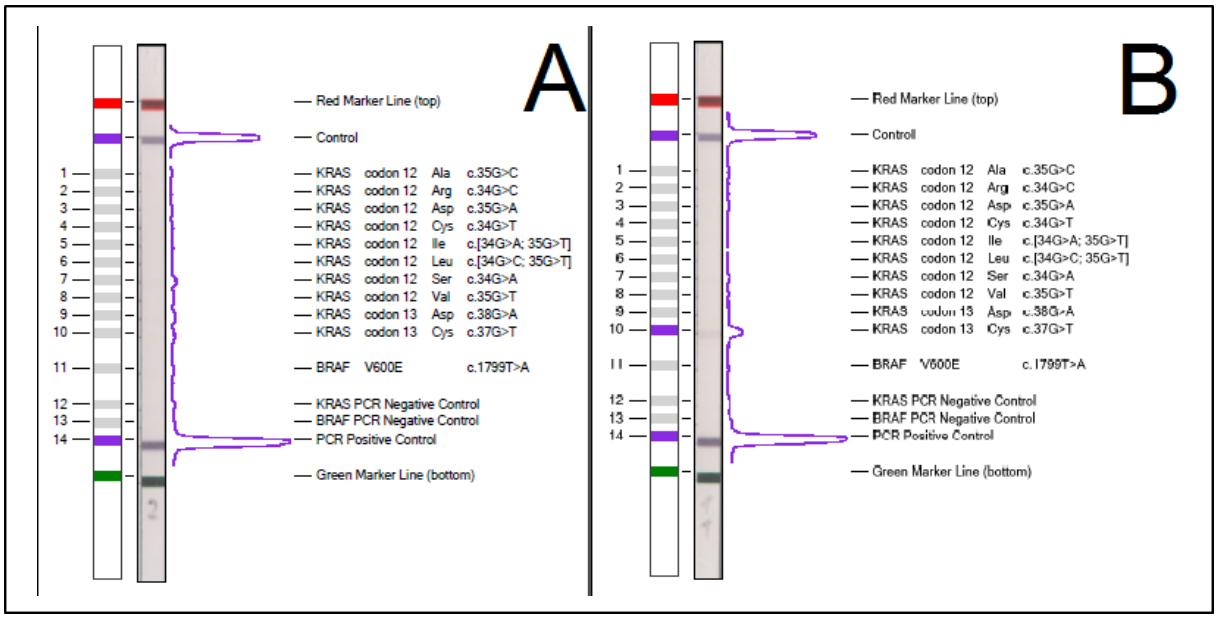

Figure 10. Example of the results generated by the KRAS-BRAF StripAssay. Red marker line on the top is a control of the conjugate solution and colour developer that should always be red. The "Control" at the second line indicates if there are enough PCR products. Line 1-11 indicate if a specific mutation appears in the sample. Wild-type DNA results in no hybridisation and mutant DNA shows a hybridisation line. A: sample with wild type *KRAS* and *BRAF* genes; B: sample number 13646 with a G to T mutation at codon 13 of the *KRAS* gene, causing an amino change from glycine to cysteine.

Mutations in *TP53* and *KRAS* genes

TP53 and *KRAS* mutations in tumour tissues

TP53 mutations were determined in tumour tissues if they are available. If there were no corresponding tumour tissues, part of the cells in ascites was used. 18 out of 24 patients presented with *TP53* mutations, while 6/24 of them were wild type. One tumour had no information regarding the histological type of the tumour (fallopian tube carcinoma) and therefore cannot be defined as type I or II (Table 6).

Additionally, *KRAS* mutations were identified in three patients (Table 6). One patient had both *TP53* and *KRAS* mutation, the other two had no *TP53* mutation, one of which being classified as Type I tumour and one as Type II tumour.

Table 6. Distributions of *TP53* and *KRAS* mutations in type I and II ovarian cancer.

	Type I	Type II
TP53 mutant	0	18 (1 *KRAS* mutation)
TP53 wild type	3 (*1 KRAS* mutation)	2 (1 *KRAS* mutation)

TP53 and *KRAS* mutations in ascites

In 15 out of the 18 patients with *TP53* mutations we examined their mutation status in ascites with FASAY (from three patients, samples were lost). In two of them, no *TP53* mutations were detected, indicating that there were no tumour cells appearing in the ascites. 4 patients presented with lower than 10% red colonies, correlating with low proportion of tumour cells and 9 patients have higher percentage of red colonies ranging from 40% to 100%, suggesting higher percentages of tumour cells appearing in the ascites.

Using the ddPCR, we could determine the exact tumour cell proportion in the ascites of 5 patients (Table 7). In pure cell cultures, consisting of either pure tumour cells or pure polygonal cells, the results of both the ddPCR and the FASAY are in concordance. If the samples were from a mixed cell culture, the results were not in concordance.

Table 7. Proportions of *TP53* mutant tumour cells in primary cell cultures / cell lines.

Cell line	Passage number	P53		Morphology
		FASAY	ddPCR	
12245	0	99%	31.64%	mixture of cells
	6	0%		polygonal adherent cells
12370	0	90%		mixture of cells
	10	100%		tumour cell clusters
	32	100%		tumour cell clusters
12636	0	99%	97.34%	mixture of cells
	3	99%	97.40%	mixture of cells
13070	0	6%	3.54%	mixture of cells
	5	4%	0.08%	polygonal adherent cells
	11	1%	0.04%	polygonal adherent cells
13088	0	4%	3.27%	mixture of cells
	4	2%	0.09%	polygonal adherent cells
	12	0%	0.00%	polygonal adherent cells
8587	2	8%	30.24%	mixture of cells
	8	90%	85.19%	adherent tumour cells surrounded by fibroblasts
	20		99.60%	adherent tumour cells
13363	0	70%	22,23%	mixture of cells
	4	60%	73.99%	mixture of cells
	7	99%	99.08%	adherent tumour cells
	10	99%	97.96%	adherent tumour cells
	19		99.96%	adherent tumour cells
13699	0	10%		mixture of cells
	3	1%		polygonal adherent cells
	3	99%		cluster of cells in supernatant
13781	0	75%		mixture of cells
	2	60%		mixture of cells
13848	0	0%		mixture of cells
	4	0%		polygonal adherent cells
13914	0	10%		mixture of cells
	3	99%		cluster of cells in supernatant
	3	1%		polygonal adherent cells

TP53 and *KRAS* mutations in primary cell cultures / cell lines

12 out of the 13 established primary cultures / cell lines were originated from materials of HGSOC patients, while one polygonal culture was from a patient with wild type *TP53* and mutant *KRAS* gene.

Primary culture was initiated with a mixture of cells, frequently including tumour cells mixed with other types of cells, like fibroblasts and mesothelial cells. In order to monitor the tumour cell purity in the culture, RNA and DNA were isolated at different passages and examined for their *TP53* mutation status with FASAY and/ or ddPCR.

In some ascites, like in the cultures from samples 13070, 13088 and 13848, no or extremely low numbers of tumour cells were present. In these cultures, polygonal cells could be separated from other types of cells easily so that pure cultures will be obtained after a couple of passages. No tumour cell lines could be cultivated from these samples.

In a couple of ascites, tumour cells appeared with very high proportions in form of clusters (samples 12370, 12636 and 13699), which could be easily separated and purified by filtration. Some of them could proliferate very quickly and reached very high passages, mostly accompanied by the death of a big number of cells and the release of the cell debris to the culture medium (cell line 12370). Some of them proliferated very quickly at the beginning but seemed to die after the initial proliferation and being overgrown from other types of cells, like in sample 12245.

In the two ascites samples number 13363 and 13914, tumour cells were present in a mixture of cells. Tumour cells adhered to culture flasks much more firmly than the polygonal cells, making it very easy to separate them by selective trypsinisation. From sample 13363, we obtained a pure tumour cell culture which was already passaged 21 times and is still proliferating. From sample 13914, we could cultivate both tumour cells and polygonal cells. The former are still proliferating at 25th passage and the latter died at the 10th passage.

Only one culture could be established from tumour tissue (sample 8587). Pure tumour cells had been obtained by continuous selective trypsinisation to deplete the fibroblasts. This primary cell line is already at the 30th passage and is still proliferating.

ddPCR confirmed that cell line 13363 and 8587 consist both of 100% tumour cells from around the 21th passage.

Types of *TP53* mutations

12 out of the 13 established primary cultures / cell lines originated from materials of patients with tumours harbouring *TP53* mutations.

All analysed *TP53* mutations are located between codon 104 and 343 of the *TP53* gene. 10 mutations are located between exon 4 to 9, which corresponds to the DNA binding domain (98-292) and two are located in exon 10 which is the tetramerisation domain (300-355).

Most of the *TP53* mutations are missense point mutations. One mutation in this region had a 3bp deletion causing a deletion of one amino acid. Two mutations were found at the tetramerisation domain, causing a reading frame shift and truncation of the p53.

Protein expression

Protein expression in cell cultures / cell lines

Both types of cells have cytokeratin expression (Figure 11).

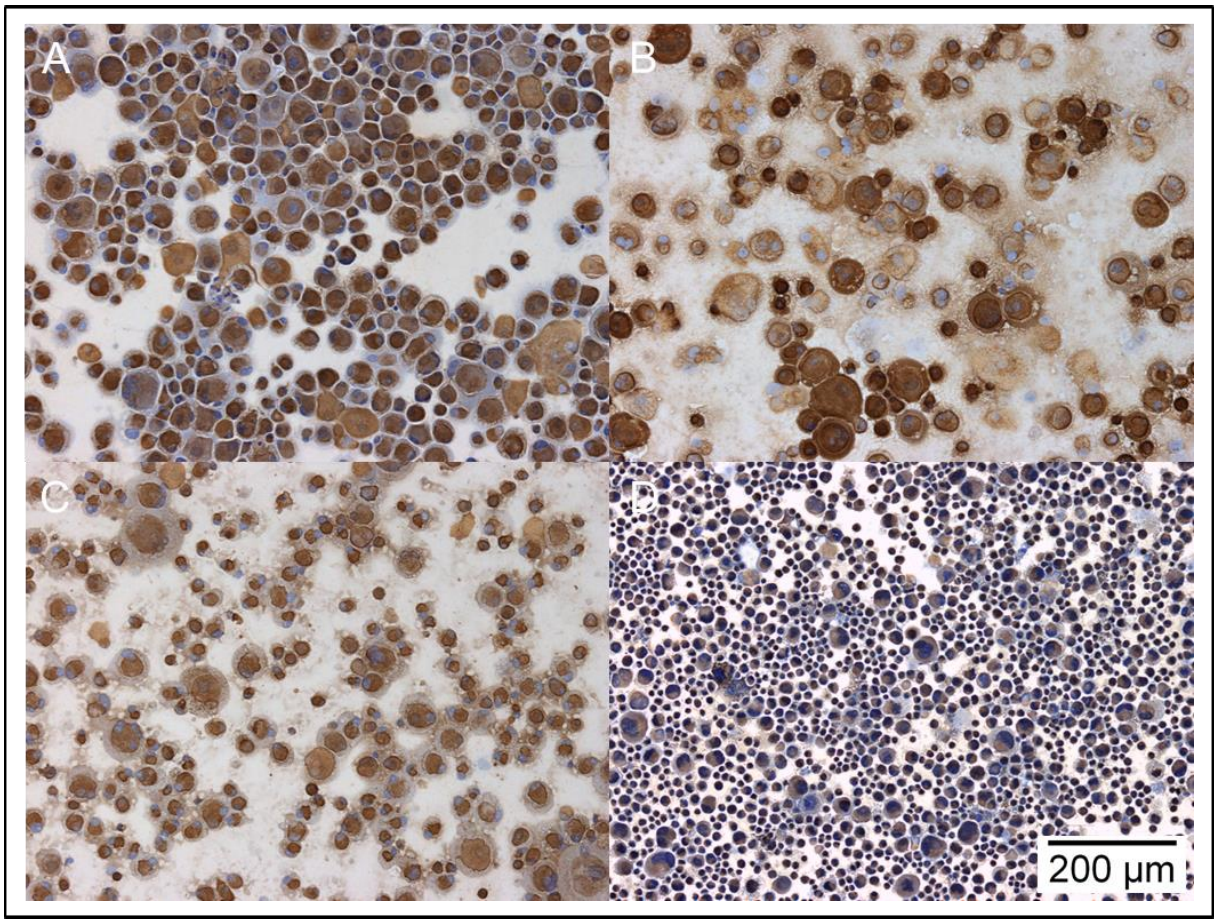

Figure 11. IHC staining of cells with pan-cytokeratin 8, 18, and 19. A, B, and C are polygonal cells from samples 13646, 12245 and 13848, respectively; D: tumour cells from sample 13914-1.

Tumour cells had EpCAM positive expression and were negative for CD44, while the polygonal methothelial cells seemed to express CD44 and had no EpCAM expression. Figure 12 shows a culture at passage 4, which had still a mixture of adherent cells. Tumour cells were relatively small with a very high nucleus / cytoplasma ration, while polygonal cells had a rather bigger cytoplasma around the nucleus. Through selective

trypsinisation, polygonal cells were gradually depleted leaving a culture with higher percentage of tumour cells at passage 7. The tumour cell clusters were obviously positive for EpCAM and negative for CD44.

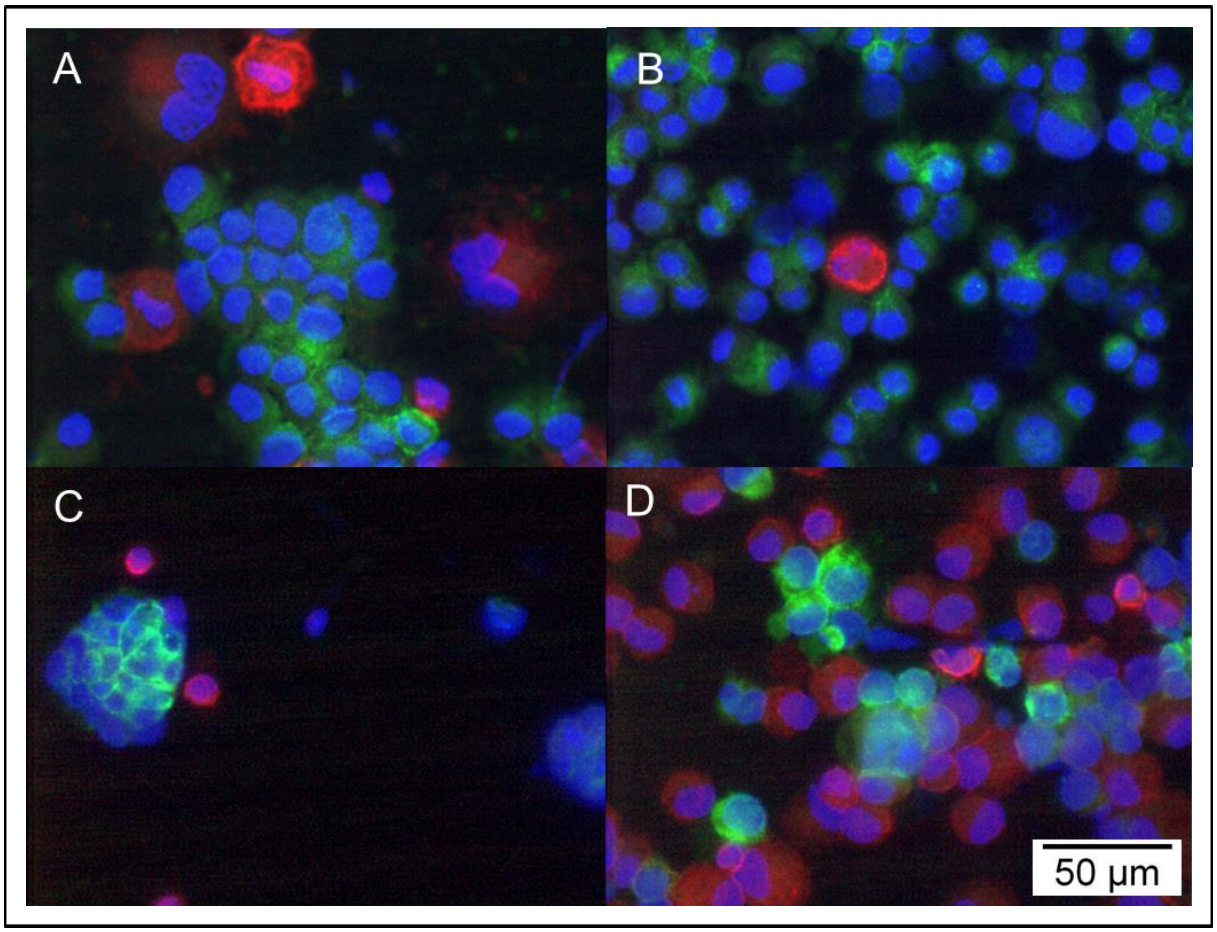

Figure 12. Immunofluorescence staining of cells with EpCAM (green) and CD44 (red) antibodies. 12A: cell culture from sample 13363 at passage 4 with only adherent cells; 12B: cell culture from sample 13363 at passage 7with only adherent cells; 12C: cell culture from sample 12636 at passage 1 with mixture of cells; 12D: sample number 12636 at passage 5 with only adherent cells.

This specific expression between the two types of cells were well demonstrated by the IHC staining of EpCAM and CD44 on the two types of cell lines derived from the same patient. While cell line 13914-1 was the tumour cell line, the 13914-2 cell line presents the polygonal cell morphology. We cultured them on chamber slides and performed a IHC (Figure 13). Both lines had cytokeratin expression. While cell line 13914-2 showed typical cell membrane staining of the cell surface marker CD44 and was negative for EpCAM, the 13914-1 cells were negative for CD44 and presented a typical membrane

staining of EpCAM. Notably, the EpCAM expression in the tumour cells had different intensity.

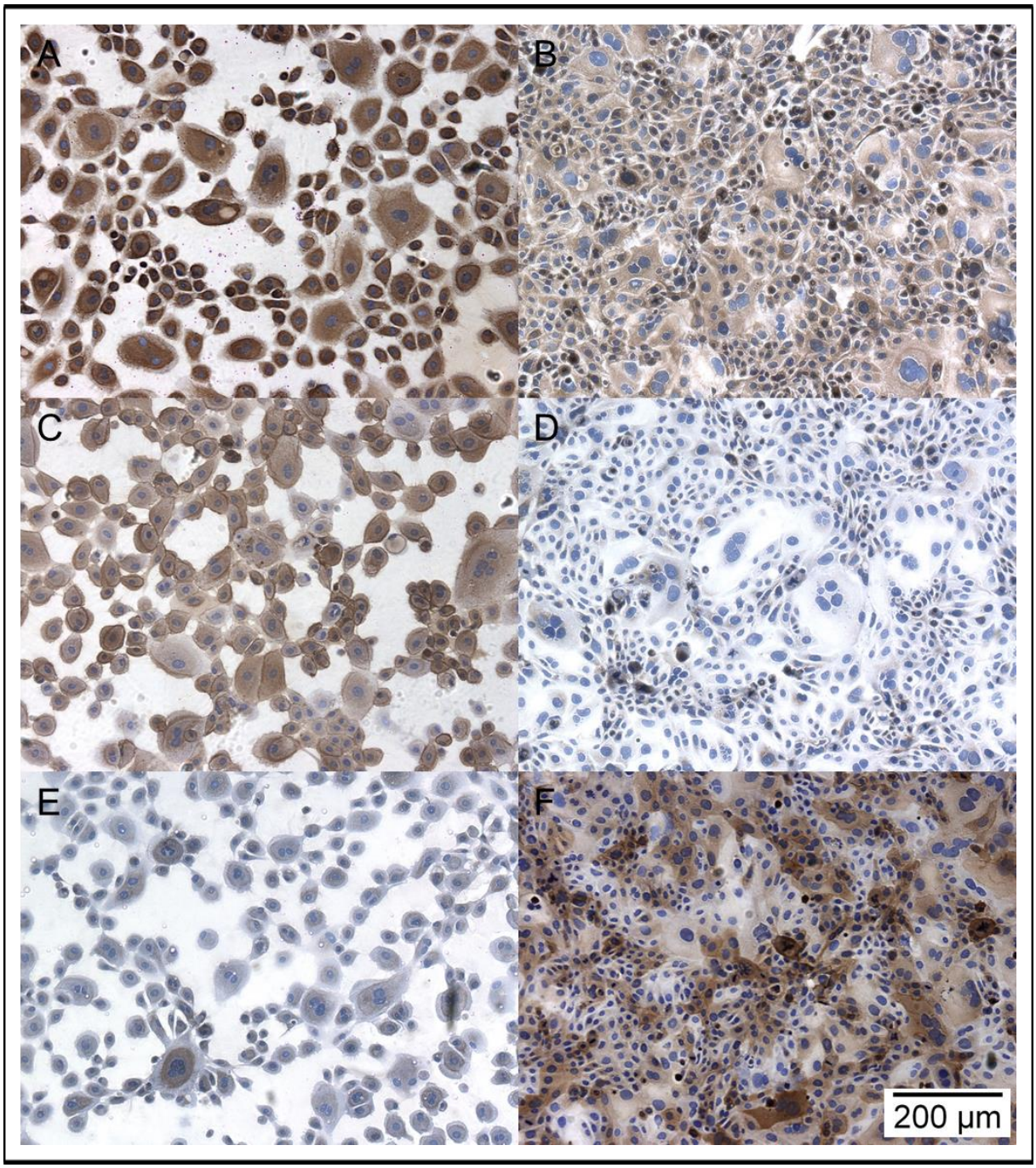

Figure 13. IHC staining of the polygonal cells 13914-2 (A, C, and E) and tumour cell lines 13914-1 (B, D, and F). A, B: staining with pan-cytokeratin 8, 18, and 19; C and D: staining with CD44 antibody; E, F: staining of EpCAM.

Calretinin was initially only examined in the paired polygonal cells and tumour cells from the same patient. It was expressed in both types of cells (Figure 14).

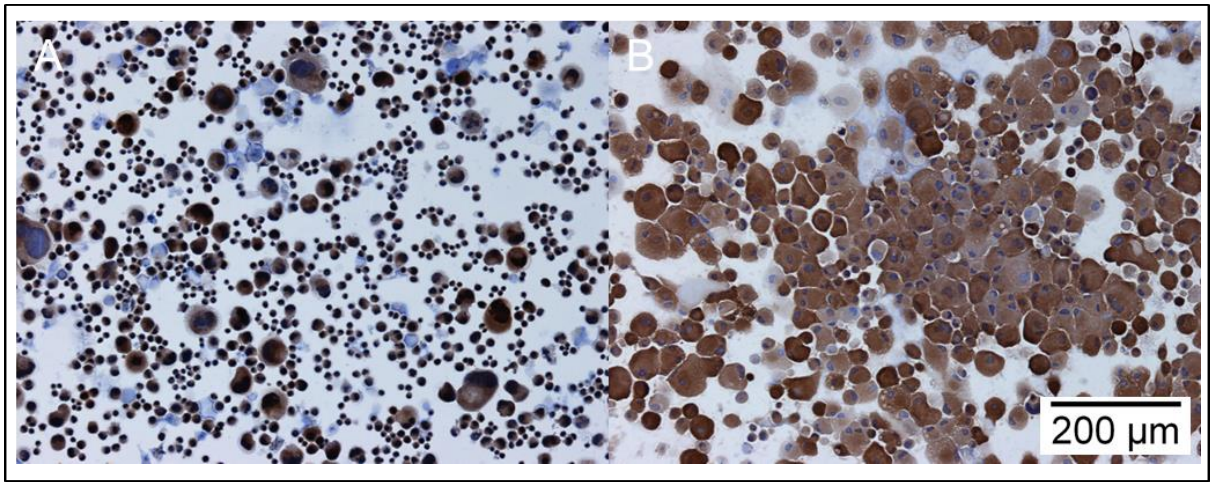

Figure 14. IHC staining of calretinin of the tumour cell line 13914-1 (A) and polygonal cell line 13914-2 (B).

Protein expression in tumour tissues

Cytokeratin staining of the tumour tissues revealed various constructions of tumours. There were some formed with relatively small solid clusters of tumour cells, tightly packed together, with stroma around each cluster (Figure 15A). Others were formed by loosely disseminated gland-like structures with one cell layer together with solid tumour clusters (Figure 15B). Sometimes tumours we observed had a sword-like structure, which were arranged in parallel (Figure 15C). While others were big solid tumours consisting of many tumour cells. In the latter, small clusters of tumour cells were found in the stroma (Figure 15D).

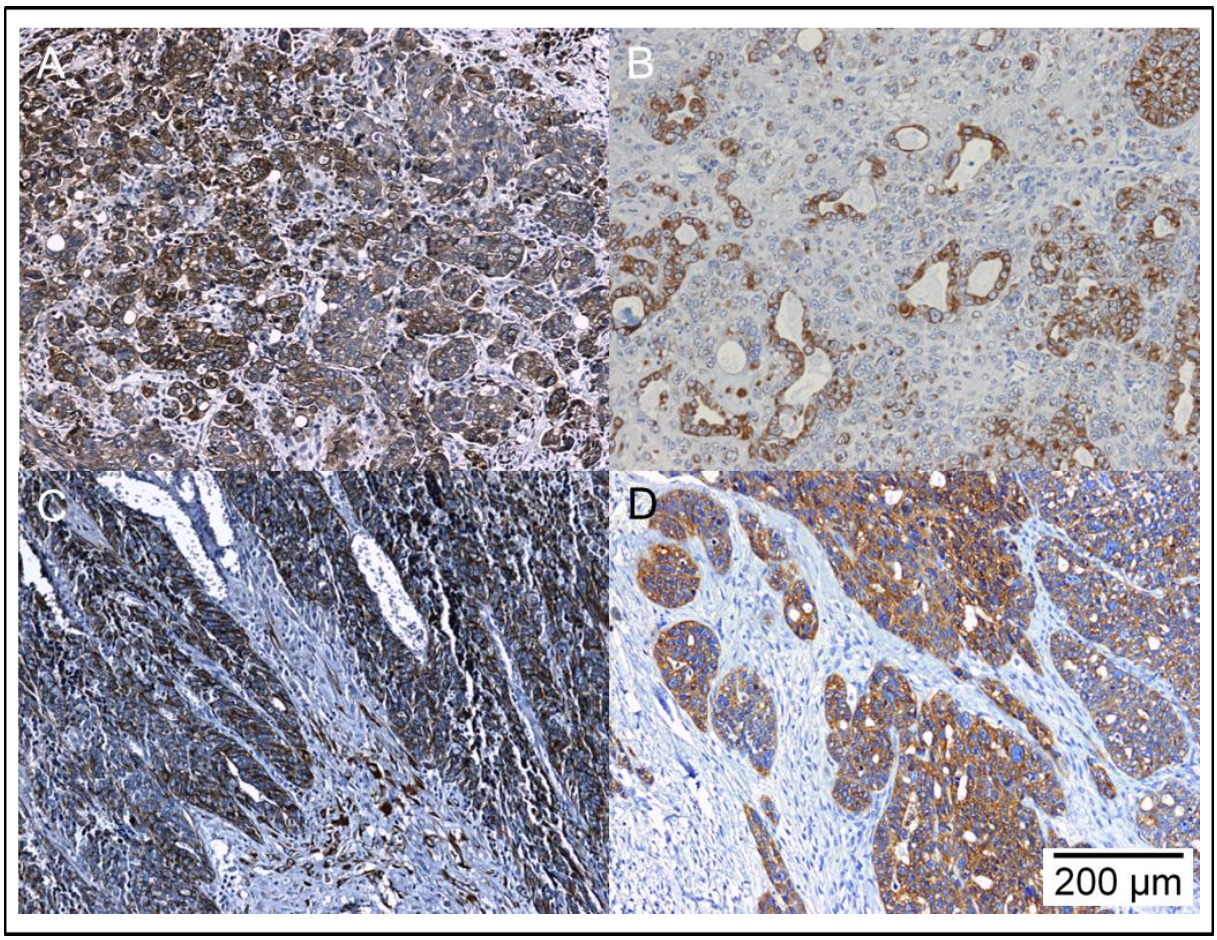

Figure 15. IHC of pan-cytekeratin 8, 18 and 19 expression in tumour tissues showing different constructions of tumours.

Almost all tumour cells had EpCAM expression (Figure 16). In the stroma, the disseminating tumour cells or cluster of cells also strongly express EpCAM.

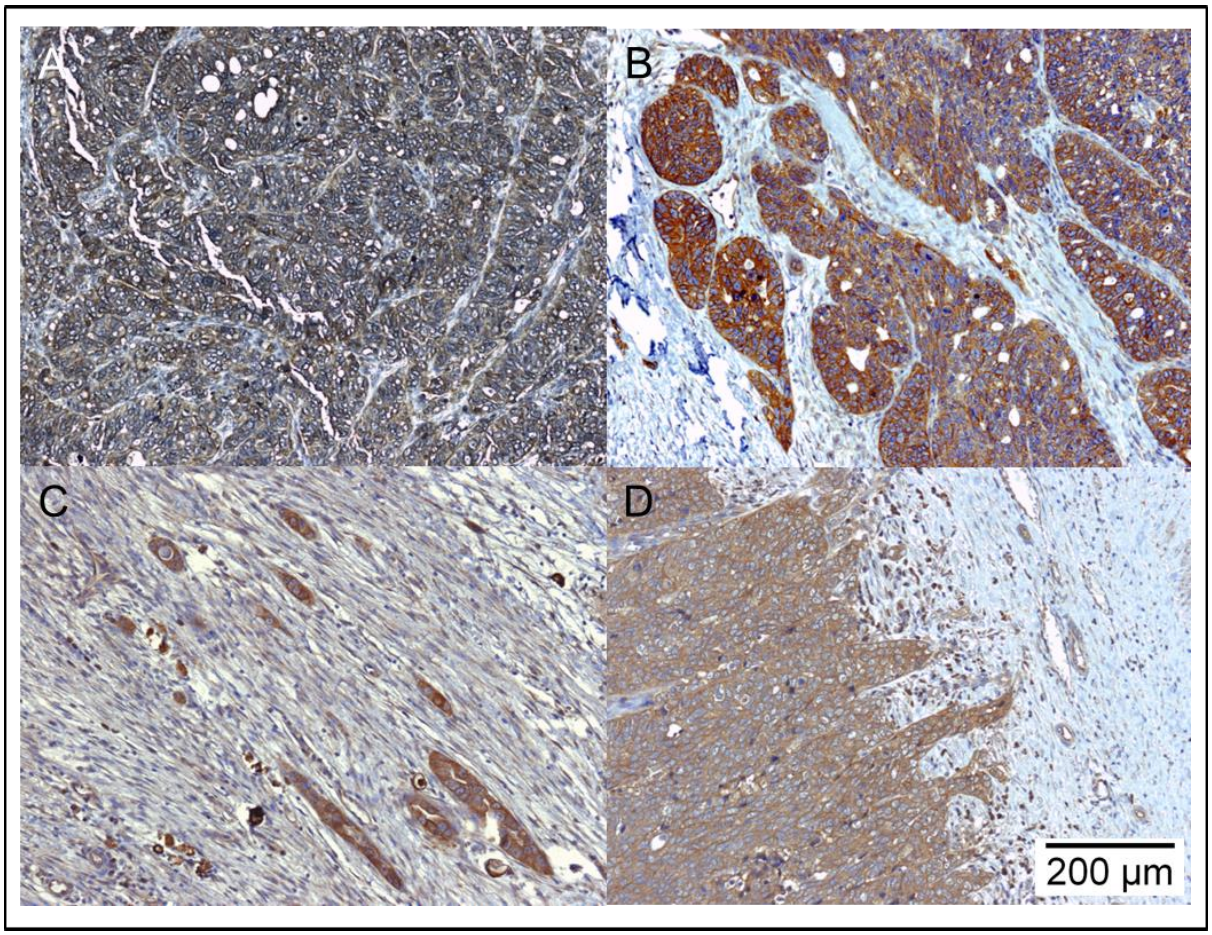

Figure 16. IHC expression of EpCAM in different tumour tissues. Not only cells in tumour mass had strong staining for EpCAM antibody also the disseminating tumour cells or small cluster of cells in stroma have strong expression of EpCAM.

Vimentin was only expressed in stromal cells but not in tumour cells (Figure 17).

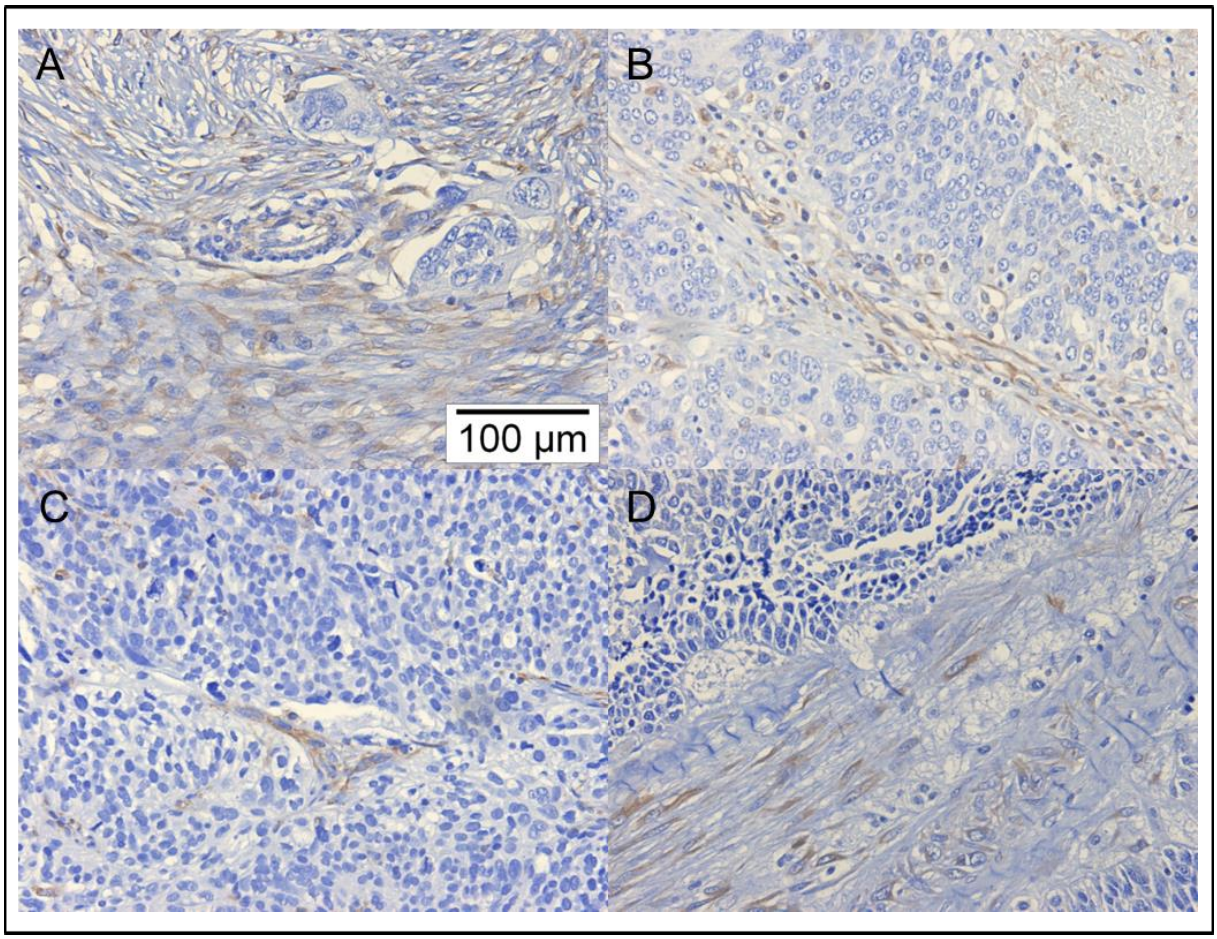

Figure 17. IHC of vimentin expression in tumour tissues staining stromal cells.

CD44 appeared only to stain the active lymphocytes around tumour cells in stroma and in the stroma between tumour cell clusters and single lymphocytes infiltrating the tumours. Tumour cells did not have any CD44 expression (Figure 18).

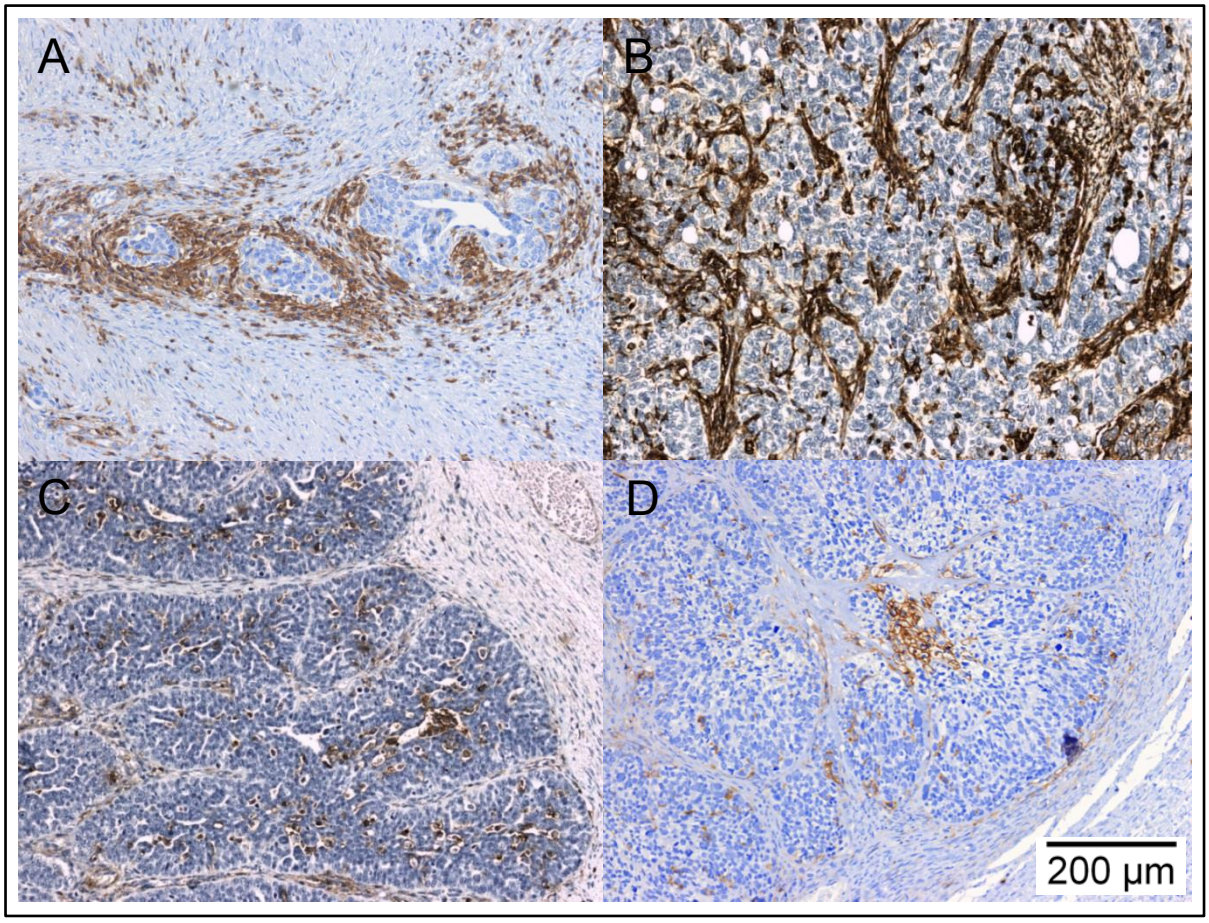

Figure 18. IHC of CD44 expression in different tumour tissues staining lymphocytes.

We notified that there were different immune reactions among the tumours. Some tumours showed a much stronger immune reaction (19A), forming lymphoids around tumour clusters while others had a rather "mild" reaction (19C).

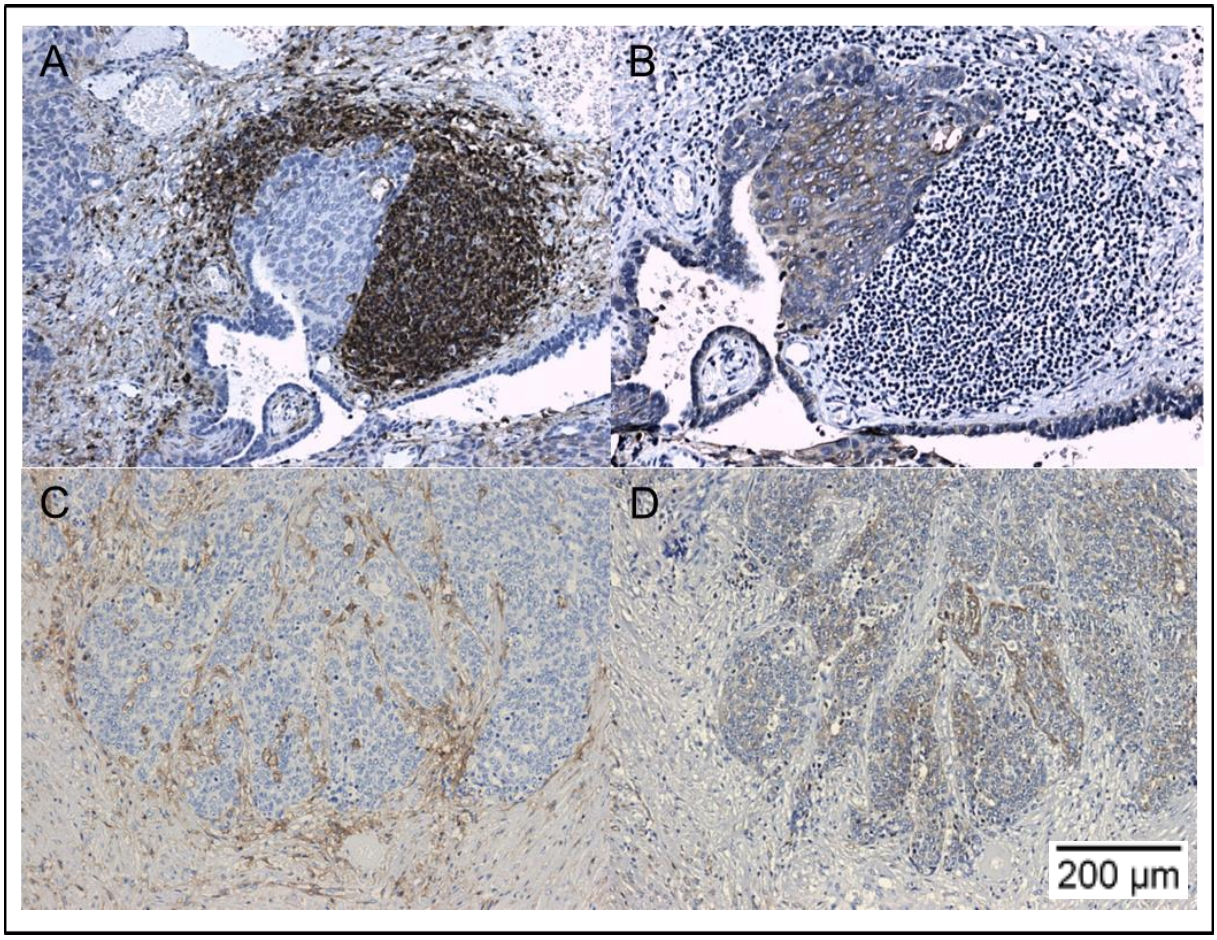

Figure 19. IHC of CD44 expression in tumour tissues showing formation of lymphoid around tumour (A,B) and a mild expression of CD44 positive cells (C,D). A, C: CD44; B,D: pan cytokeratin 8, 18, 19.

Calretinin was only expressed in some cells of the tumour tissues. As shown in figure 20, it was mostly expressed in stromal cells whereas in some tumour tissues, calretinin was only partially expressed. Even in the same tumour clusters, some cells were stained positive, while others were not. Additionally we found a positive expression of calretinin in high differentiated tumour parts as well as in cells surrounding micro-vessels.

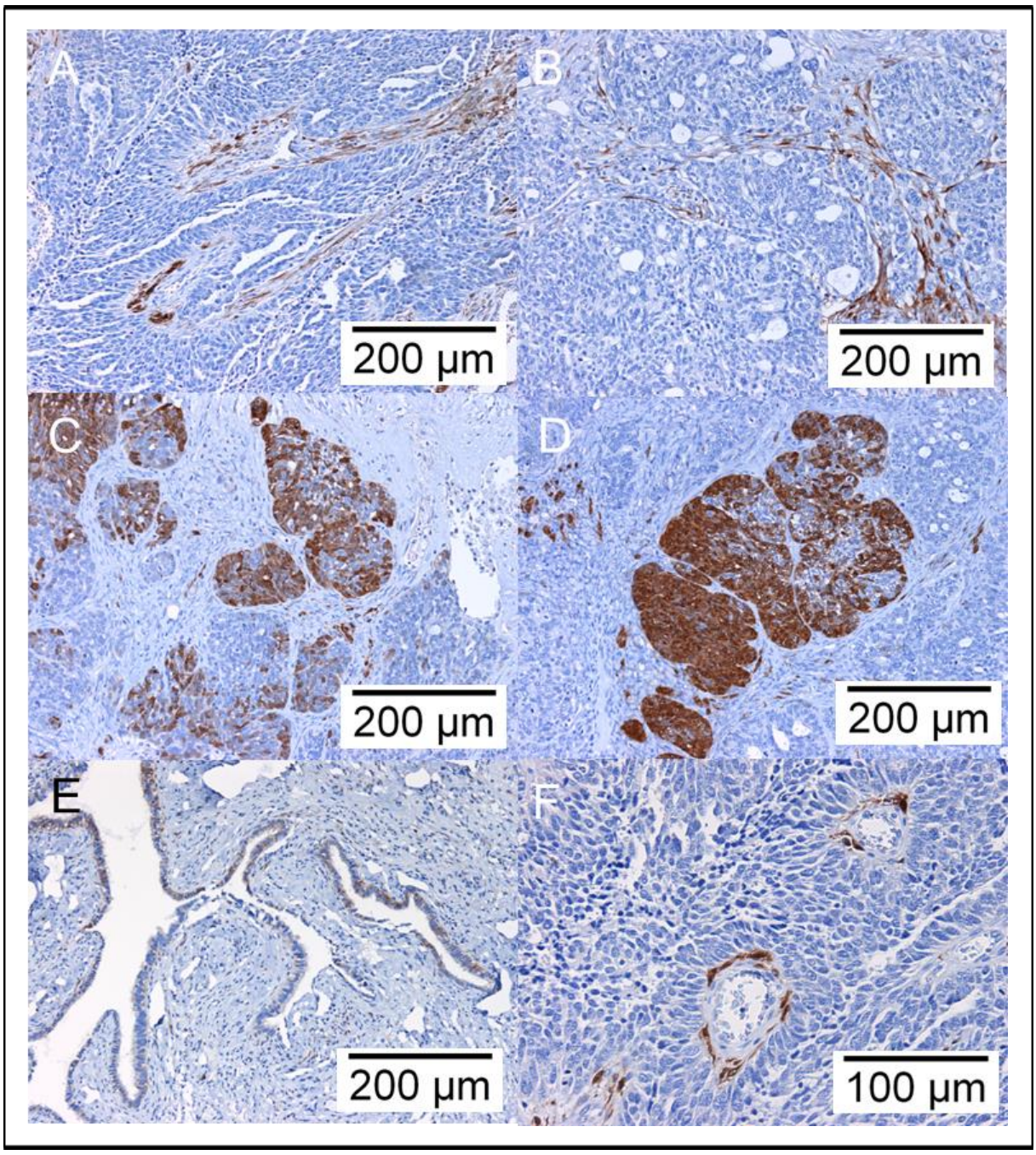

Figure 20. IHC expression of calretinin in tumour tissues. A, B: expression in stromal cells; C, D: expression in tumour cells; E: expression in high differentiated tumour parts; F: expression in cells surrounding the micro-vessels in tumours.

Discussion

Models representing HGSOC are very important for ovarian cancer research. In the Helene Harris Memorial Trust Forum on Ovarian Cancer meeting held in 2011, findings in basic, translational and clinical research have been summarized and discussed by leading researchers in this field. Under the recommendations proposed for further research, better experimental models were required as one of the most important issues (Vaughan, Coward et al. 2011). As different histological types of ovarian cancers have been confirmed as being derived and driven from different molecular mechanisms, it is of the outmost importance to have cell line models, which closely resemble the various disease.

During our current work, we have established 12 primary cell cultures, 7 of these were generated from either ascites or tumour tissues of high grade serous ovarian cancer patients. 4 of them were confirmed to be pure tumour cells and were passaged over 20 times. Cells from these four lines harbour the same *TP53* mutation as presented in their corresponding tumour tissues. Protein expression analyses demonstrated that the cell lines had the same expression pattern of cytokeratin, EpCAM, CD44, calretinin and vimentin as the corresponding tumour tissues. These cell lines satisfy the urgent need of ovarian cancer research and present optimal models of HGSOC.

Ovarian cancer is being recognized as a disease with distinct molecular backgrounds (Shih Ie and Kurman 2004). 75% of the epithelial ovarian cancer is a high-grade serous type making it a very important research target. However, most of our knowledge on ovarian cancer was generated from cell line models, which were not well defined and molecularly well characterized. A recent work indicated that the overwhelming number of publications regarding ovarian cancer cells derived from cell lines, which were most unlikely high grade serous (Domcke, Sinha et al. 2013). The tumour cell lines established in this current research are derived from high grade serous epithelial ovarian cancer, making them attractive and useful experimental models.

Among the 7 primary cultures / cell lines, two major growth patterns have been observed. One type of cells appears mostly in clusters. They have a very quick proliferation, during which many cells also die leaving large amounts of cell debris in the

culture. The adherent cells had different growth rates. While some of them had a doubling time of 3-4 days, others need some 7-10 days. The long duration of the doubling time is not only a result of slow growth, but also a consequence of cell death during growth and proliferation. We observed that in these lines, some cells enlarged enormously in their size and attached to the culture flask over huge areas. These heterogeneous growth patterns of the tumour cells may reflect different molecular processes. To analyse the expression profiles of these cells and associate them with the clinical follow-up of the disease, it will certainly help us to understand the mechanisms underlying some important processes like recurrence or chemoresistance.

We also established primary cultures of some polygonal cells, which may possible be derived from the mesothelium in the peritoneal cavity. Some HGSOC does not cause the production of ascites, while others do. It was reported that the survival rate of patients without ascites is much better than for patients with ascites (Puls, Duniho et al. 1996). Our study indicated that in the ascites, not only tumour cells appeared in varying amounts, ranging from zero to a very high percentage of 99%, but also the mesothelial cells originated from the lining of the peritoneal cavity were released in various amounts. We assume that tumour cells may exhibit an unequal potential in degrading the extracellular membrane to grow and to invade the surrounding tissues. The release of the mesothelial cells might be an effect reflecting characteristics of the tumour. A recent publication indicates that ovarian cancers might be classified into two groups, one of which has an activated extracellular matrix driven program, IFN signalling and immune evasion (Gardi, Deshpande et al. 2013).

Cultured tumour cells had a positive protein expression of cytokeratin 8, 18 and 19 and EpCAM but not for CD44 and vimentin, which is in concordance with their corresponding tumour tissues. We also examined the calretinin expression of the tumour cells. Interestingly, the expression of calretinin was very different in tumour tissues. Some had a positive staining, while some others did not. In the established cell lines, we have examined two cell lines, which represented tumour cells and mesothelial cells, and were derived from the same patients. Not only had the polygonal mesothelial cells a positive calretinin expression, but also the tumour cells were positive, which was also found in the corresponding tumour tissue. So far, calretinin was mostly used as a mesothelial marker. There were fewer publications regarding its expression in tumour

tissues, especially in HGSOC. It will be interesting to further investigate the expression and role of this marker in ovarian cancer.

We have found different types of *TP53* mutations in tumour tissues, most of them are within the DNA-binding-domain. As mentioned before, different types of mutations can trigger distinct molecular processes. It is of the highest interest to understand the pathways that each mutation or each type of mutation has induced in the cells affecting the development and progression of the tumours. IHC has revealed different pattern of tumour constructions and different disseminating types, which might be associated with the types of the *TP53* mutations. Based on the current works, expression profiling analyses of the tumour cells are ongoing, from which specific molecular pathways related to certain tumour characteristics are expected to be obtained.

In this study we used three methods to identify, detect and quantify *TP53* mutations, i.e. FASAY, direct sequencing, and ddPCR, respectively. The FASAY method is a time consuming method but is necessary to identify *TP53* mutations in single samples. As soon as the mutation is defined, it can be detected by direct sequencing, which is a quick and easy method. However, it lacks the detection sensitivity. ddPCR is a very advanced technology, which can detect very low copy numbers of the targeted gene and deliver precise results. For each mutation, a unique system must be established and optimized, making this method very expensive. FASAY gives readout of the percentage of red colonies, which corresponds to the cDNA copies reverse transcribed from mRNA of tumour cells. If a sample consists of cells with pure genotype, i.e. only the mutant or the wild-type gene, the FASAY results were in accordance with the results generated by the ddPCR. When mRNA was isolated from a mixture of cells with both wild-type and mutant genotype, the percentage obtained from FASAY did not represent the proportion of cells with a certain genotype because mRNA of *TP53* is transcribed in different amounts in different cells. In addition, mutant *TP53* transcripts seemed to be more stable than the wild-type. These might be the main reasons to explain why FASAY cannot be used for quantification of tumour cells in a mixed culture.

For decades, ovarian cancer was considered to be “one” disease. Since the publication of the Type I and Type II theory of ovarian cancer (Shih Ie and Kurman 2004), this cancer becomes more and more a multiple disease with heterogeneous molecular drivers. Our results show, that even among the HGSOC, distinct biological processes might be hidden.

Different morphologies, growth rates and disseminating pattern might be consequences of each unique *TP53* mutation and might be an indicator for the tumour progression, eventually for the clinical outcome of the patients. Studying the mechanisms underlying the heterogeneity of the tumour is the basis to understand the recurrence and chemo-resistance of ovarian cancer cells and to help develop new and effective therapies against this disease. The establishment of well-defined cell lines paved the first stone of the avenue leading us to the final goal of research to cure the ovarian cancer patients.

In summary, we established tumour cell lines, which are derived from ascites or tumour tissues of patients with high grade serous ovarian cancer. They harbour the same *TP53* mutation as their corresponding tumours and have the same protein expression of cytokeratin, CD44, EpCAM, vimentin and calretinin. They can serve as optimal models for investigating mechanisms underlying HGSOC.

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I would like to thank my supervisor Prof. Dr. Dan Cacsire Castillo-Tong for supporting me and my work every day of the year. I also want to express my deepest gratitude towards my team leader Prof. Dr. Robert Zeillinger and my colleagues that I have worked with: Andrea Wolf, Eva Schuster, Elisabeth Maritschnegg, Stefanie Aust and Grazyna Dudek. At the end, I would especially like to thank my family for encouraging me with my work. Words cannot express how grateful I am. Completing my Master would not have been possible without them.

Index A

Curriculum vitae

Caroline KREUZINGER

E D U C A T I O N

2011 – 2013	**University of Vienna** **Master in Molecular Biology – Molecular Medicine** First year in Epigenetics and Cancer, Stem Cells, Cloning, Molecular Medicine, Spectroscopy, Cell biology and Patent law Second year in Ovarian Cancer Research	Vienna, Austria
2008 – 2011	**University of Applied Sciences – FH Campus Wien** **Bachelor in Molecular Biotechnology** Completed the First year in Biology, Chemistry (2 labs), Molecular Biology and Genetics, Cell biology (lab), Biochemistry and Scientific English Completed the Second year in Biochemistry Microbiology, Molecular Biology (lab), Chemistry, Cell Biology (lab) GMP&GLP, Histology, Molecular biology (lab), Developmental Biology and English Science and Career Completed the Third year in model organisms, human physiology, Immunology, Marketing & Sales, Clinical Studies, Product Management, Tissue Engineering, Organic chemistry (lab), bachelor thesis with focus on p53	Vienna, Austria
1995 – 2008	**Lycée Français de Vienne** French Baccalauréat, Specialized in Sciences: Biology, Chemistry and Physics Matura – Reifeprüfung, graduated with honours	Vienna, Austria

E X P E R I E N C E

Sep-Oct 2012 *2 months*	**Project - IQGAP1: a new therapeutic target for ovarian cancer patients with poor survival – Lowy Cancer Research Institute University of NSW** Individual laboratory work Cell culture (serous ovarian cancer and normal control cell lines) Drug treatment (IC50) RNA and Protein expression – Western Blot	Sydney, Australia
July 2011 *1 year*	**Laboratory work at the AKH – Allgemeines Krankenhaus - Wien** Laboratory Assistant	Vienna, Austria

Sample analysis (DNA-Isolation, PCR, Sequencing)
P53 analysis
Working in a group of 5 members
Involved in Clinical Studies

A D DI T I O N A L

Languages	Fluent in **German, French, English** Other Languages: **Latin** (Five years language course – 2002/07)
IT Skills	MS Office **(Very good)**. Databases: clustalW, blast. Analysing Programs: ABI Prism™ 310 Collection, Auto Assembler 2.1, QuantaSoft
Societies and interest	Extroverted, open-minded Organisational skills Enjoy cooking, hiking and travelling with my dog Driving licence B

Index B

RT: room temperature
min: minute
sec: second
g: g force
°C: temperature Celsius
µm: micrometre
µg: microgram
ml: millilitres
mg: milligram
cm: centimetre
W: watt

Materials and Solutions	Company (or composition)
Biowizard Golden GL-130, Class II	KOJAIR
PBS	Life Technologies
Trypsin	
DMEM	
Penicillin Streptomycin	
FKS	
DMSO	Sigma Aldrich
Collagenase	Life Technologies
Histopaque® 1077	Sigma Aldrich
Cell Strainer 70µm, 100µm mesh	BD Biosciences
Fibroblast Growth Factor	Life Technologies
Standard MF-Millipore Membranes, white, plain	Millipore, Germany
Mikro-Dismembrator U	B.Braun, Biotech International
AllPrep DNA/RNA Mini Kit	Qiagen, Germany
BioPhotometer	Eppendorf, Germany
MPG mRNA Purification Kit	PureBiotech, USA
Omniscript RT Kit	Qiagen, Germany
Yeast strain yIG397	
linearised pRDI-22 vector	
sonicated salmon sperm DNA as carrier DNA	
Agar plates/medium for yeast strain alone (with Bacto agar/ w/o Bacto agar)	(1)
Agar plates/medium for transformed yeast (with Bacto agar/ w/o Bacto agar)	(2)
Bacto Yeast Extract (1)	BD Biosciences
Bacto Peptone (1)	
Difco Yeast Nitrogen Base w/o Amino Acids (2)	
Bacto Agar (1+2)	
Dextrose (1+2)	Sigma Aldrich
Aα-Solution: Amino acid solution with: Arg, Val, Phe, Met, Thr, Glu (1+2)	Solids from Sigma Aldrich dissolved in ddH2O

TRP 1% (1+2)	
HIS 1% (1+2)	
Adenin 0.5% (1+2)	
Primer P3, sense	5'-ATT-TGA-TGC-TGT-CCC-CGG-ACG-ATA-TTG-AAC-3'
Primer P4, antisense	5'-ACC-CTT-TTT-GGA-CTT-CAG-GTG-GCT-GGC-GTG-3'
Primer C1, SE	5'-CCT-CCT-CAG-CAT-CTT-ATC-3'
Primer C1, AS	5'-AAG-AAG-TGG-AGA-ATG-TCA-G-3'
Primer C2, SE	5'-CCA-AGC-AAT-GGA-TGA-TT-3'
Primer C2, AS	5'-TAG-TGG-ATG-GTG-GTA-CAG-TC-3'
GFX PCR DNA	Amersham Bioscience, Uppsala, Sweden
Gel Band Purification Kit	
Poly-L-Lysine Coated Microscope Slides	Thermo Scientific
Centrifuge Universal 16A	Hettich
Formaldehyde 4% (dilute with PBS)	Sigma Aldrich
1xPBS	Life Technologies
0,5% Triton X in PBS	Sigma Aldrich
PBS-Tween: 2.5ml Tween ad 2000ml PBS	Sigma Aldrich
Primary Antibody Cytokeratin pan 8,18,19, A45-B/B3	AS Diagnostik
Primary Antibody Vimentin, ab125089	abcam
Primary Antibody EpCAM, ab32392	abcam
Primary Antibody CD44, 20282	ProMab
Primary Antibody Calretinin, 180211	Life Technologies
FLEX Negative Control Mouse Cocktail, IS750	Dako
Antibody diluent	Dako
Dako Ultra V Block	
Dako IHC Staining Kit	
Southern Biotech Fluoromount-G	Southern Biotech
DAPI (4',6-diamidino-2-phenylindole)	Life Technologies
Alexa Fluor® Dyes	Invitrogen
Oil droplets (ddPCR Droplet Generation Oil)	QuantaLife, Biorad
Droplet generator (QX100 Droplet Generator)	Biorad
Droplet reader (QX100 Droplet Reader)	
Software (QuantaSoft)	QuantaLife, Biorad
Microscope Olympus BX50	Olympus
Soft Imaging System CC12	

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