Mitomycin C sensitivity and chromosomal alterations in *BRCA2* mutated cells

Hörður Bjarnason

Thesis for the degree of Master of Science
University of Iceland
Faculty of Medicine
School of Health Sciences

HÁSKÓLI ÍSLANDS

Litningabreytingar af völdum Mitomycin C í *BRCA2* arfblendnum frumum

Hörður Bjarnason

Ritgerð til meistaragráðu í Líf- og læknavísindum

Umsjónarkennari: Jórunn Erla Eyfjörð

Leiðbeinandi: Sigríður Klara Böðvarsdóttir

Meistaranámsnefnd: Sigríður Klara Böðvarsdóttir, Jórunn Erla Eyfjörð og Margrét Steinarsdóttir

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Hörður Bjarnason

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Supervisor: Jórunn Erla Eyfjörð

Instructor: Sigríður Klara Böðvarsdóttir

Masters committee: Sigríður Klara Böðvarsdóttir, Jórunn Erla Eyfjörð and Margrét Steinarsdóttir

Faculty of Medicine

School of Health Sciences

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Ágrip

Fanconi blóðleysi (FA) er meðfæddur erfðasjúkdómur sem orsakast af óvikjun beggja samsæta í einu af 15 þekktum FA genum. *FA-D1* er eitt þessara gena og er í raun sama gen og *BRCA2*. *FA-D1/BRCA2* genið er þátttakandi í mikilvægum frumulíffræðilegum ferlum s.s. þáttapörunarviðgerðum (e.homologous recombination) á tvíþátta DNA brotum, verndun nýmyndaðs erfðaefnis við stöðvaðar eftirmyndunarkvíslir og nýverið hafa vísbendingar bent til þátttöku þess í viðhaldi telomere raða á litningaendum. Kímlínustökkbreytingin *999del5* í *BRCA2* geninu er þekkt í íslenska þýðinu og eykur stórlega líkur á brjóstakrabbameinum auk annarra gerða krabbameina.

Markmið þessarar rannsóknar var að greina og bera saman litningagalla (e.chromosomal abnormalities) og galla í litningaendum í frumulínum með mismunandi *BRCA2* arfgerðir. Eitilfrumulínur úr mönnum með mismunandi *BRCA2* arfgerðir, þ.e. óstökkbreyttar $BRCA2^{+/+}$, arfblendnar $BRCA2^{+/-}$ frumurlínur og ein arfhrein $BRCA2^{-/-}$ FA-D1 frumulína, voru ræktaðar og greindar ýmist ómeðhöndlaðar eða eftir meðhöndlun með mítómýsín C. Mítómýsín C er DNA víxltengjandi (e.cross-linking) lyf sem notað er til að meta DNA viðgerðahæfni fruma. Í verkefninu var borið saman hvort munur væri á litningagöllum milli frumulína eftir arfgerðum. Litningar voru heimtir og litningagallar greindir á metafösum eftir Leishman litun. Gallar í litningaendum voru greindir með PNA-FISH aðferð, einnig á metafösum. Niðurstöðurnar sýna að arfhreina $BRCA2^{-/-}$ frumulínan sýnir tölfræðilega marktæka aukningu á litningagöllum (DNA brotum og endatenging litninga (e.chromosomal end-joining)) samanborið við arfblendnar $BRCA2^{+/-}$ og/eða óstökkbreyttar $BRCA2^{+/+}$ frumulínur eftir meðhöndlun með Mitomýsín C. Engin marktækni var hins vegar í litningagöllum milli óstökkbreyttu $BRCA2^{+/+}$ og arfblendnu $BRCA2^{+/-}$ frumulínanna. Greiningar á litningaendagöllum sýndu hins vegar tölfræðilega marktæka aukningu á göllum, s.s. vöntun á telomere-merkjum á litningaendum og aukningu í telomere-merkjum inn á litningum, í arfblendnum *BRCA2* frumum samanborið við óstökkbreyttar frumur. Einnig var marktæk aukning á göllum í arfhreinu $BRCA2^{-/-}$ frumulínunni samanborið við þær arfblendnu, þ.e. stighækkandi aukning í göllum á litningaendum eftir *BRCA2* arfgerð. Niðurstöðurnar styðja að *BRCA2* hefur hlutverki að gegna í viðhaldi erfðaefnis og stuðlar að viðhaldi litningaenda. Niðurstöðurnar benda sömuleiðis til að þátttaka *BRCA2* í viðhaldi telomere-raða á litningaendum sé viðkvæmari en þátttaka þess í þáttapörunarviðgerðum þar sem *999del5* kímlínustökkbreytingin hefur meiri áhrif á viðhald litningaendana en almennt á litningagalla. Því virðist gæta arfstakra áhrifa (e. haploinsufficiency) *BRCA2* í viðhaldi telomere-raða á litningaendum.

Abstract

Fanconi anemia (FA) is a genetic disorder caused by biallelic mutations in one of the 15 known FA genes. *FA-D1* is identical to *BRCA2* and is in fact the same gene. The *FA-D1/BRCA2* gene is known to be involved in cellular pathways such as homologous recombination repair of double strand DNA breaks, stabilization of nascent DNA strands at stalled replication forks and also in telomere maintenance and integrity. In the Icelandic population a germline mutation *999del5* in the *BRCA2* gene is known to increase breast cancer predisposition as well as other cancer types.

The aim of this study was to analyze differences in chromosomal stability and telomere maintenance between different *BRCA2* genotypes. Human lymphocyte cell lines of different *BRCA2* genotypes (wild-type $BRCA2^{+/+}$, heterozygous $BRCA2^{+/-}$ and homozygous $BRCA2^{-/-}$ of FA-D1 origin) were used and analyzed for chromosomal stability either untreated or after treatment with Mitomycin C, a DNA crosslinking agent. Chromosomes were harvested and chromosomal abnormalities analyzed using solid Leishman staining on metaphase spreads and dysfunctional telomeres were analyzed using PNA-FISH method, also on metaphase spreads. Results showed that the homozygous $BRCA2^{-/-}$ cell line showed significantly increased DNA breaks and chromosomal end-to-end fusions compared to the heterozygous $BRCA2^{+/-}$ and/or wild-type $BRCA2^{+/+}$ cell lines after Mitomycin C treatment, but no difference was found between the wild-type $BRCA2^{+/+}$ and the heterozygous $BRCA2^{+/-}$ cell lines. Significant stepwise increase was found in telomere dysfunction after Mitomycin C treatment in regard to the *BRCA2* genotype, starting from the wild-type *BRCA2* cell lines with the lowest frequency of telomere signal loss from chromosome ends and interstitial telomere sequences to the highest frequency in the homozygous *BRCA2* cell line. These results support that *BRCA2* has a role in keeping genetic integrity and telomere maintenance, indicating that BRCA2s role in telomere maintenance might be of a more delicate nature than homologous recombination repair of DSB since the *999del5* germline mutation seems to have a greater impact on telomere dysfunction than chromosomal abnormalities. Therefore possible dysfunctional telomere maintenance among *BRCA2* mutation carriers may be resulted from BRCA2 haploinsufficiency.

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ALT	Alternative lengthening of telomeres
ATR	Ataxia telangiectasia
BFB	Breakage fusion bridge
bp	Base pair
BRCA1	Breast cancer gene 1
BRCA2	Breast cancer gene 2
CEF	Chromatid end fusion
CGH	Comparative genomic hybridization
DAPI	Diamidinophenylindole
DNA	Deoxyribose nucleic acid
DSB	Double strand break
ECTR	Extrachromosomal telomeric repeats
ER	Estrogen receptor
FA	Fanconi anemia
FA-D1	Fanconi anemia gene D1
FBS	Fetal bovine serum
FISH	Fluorescence in situ hybridization
FITC	Fluorescein isothiocyanate
HER2	Human epidermal growth factor receptor 2
HR	Homologous recombination
ITS	Interstitial telomeric sequences
KCl	Potassium chloride
LOH	Loss of heterozygosity
MEF	Mouse embryonic fibroblasts
MMC	Mitomycin C
MTS	Multi telomere signal
NER	Nucleotide excision repair
NHEJ	Non-homologous end joining
PR	Progesterone receptor
PNA	Peptide nucleic acid
RPA	Replication protein A
SEM	Standard error of means
SSC	Saline-sodium citrate

ssDNA	single stranded DNA
TIF	Telomere dysfunction induced foci
T-SCE	Telomere sister chromatid exchanges
TSG	Tumor suppressor gene

Foreword

Heterozygous mutations in the *BRCA2* gene are known to increase cancer susceptibility in carriers and the loss of the remaining wild-type allele by somatic mutations used to be thought as essential for tumor initiation (Berger *et al*., 2011). In my BSc thesis, a quantitative real time TaqMan PCR method was developed to estimate the relative loss of the wild-type allele in DNA samples derived from breast-, prostate- and pancreatic cancers in *BRCA2*^{999del5} mutation carriers and mammary epithelial cell lines (Bjarnason, 2009). The method was based on allele specific primer pairs making it possible to quantitatively amplify the *BRCA2* wild-type allele and the mutated *BRCA2*^{999del5} allele in separate PCR using the same TaqMan probe. The proportion of the wild-type allele versus the *999del5* allele was then calculated to estimate the loss of heterozygosity (LOH) in each tumor. The results showed that little or less than 50% of wild-type allele loss was present in about half of all cancers analyzed questioning whether LOH really is the initial step of tumor formation in all cases (Bjarnason, 2009). If that is the case, the cancer predisposition would result from a haploinsufficient role of the *BRCA2* gene which leads us to the specific aims of this MSc-thesis as discussed later.

The results have contributed to three published papers all attached to this thesis in appendix 1 -3. One showed the correlation between the *BRCA2* LOH and different tumor histology in seven human pancreatic cancers that matched the histology of pancreatic cancers in a murine model with controlled LOH of the *BRCA2* gene. This resulted in strong evidence that somatic mutations of the remaining *BRCA2* allele is not necessarily needed for pancreatic carcinogenesis (Skoulidis, 2010. [Appendix paper 1]). In the second paper the *BRCA2* LOH is significantly correlated to a strong proliferative marker, Ki67, in luminal type breast cancers suggesting that LOH might be involved as a late event in tumor progression rather than tumor initiation in luminal type breast cancers (Stefansson et. al, 2011. [Appendix paper 2]. In the third paper, cell lines derived from *BRCA2 999del5* carriers used in the study and breast tumors also from *BRCA2* mutation carriers were analyzed for LOH and presented as supplementary data. The study presents telomere dysfunction in breast tumors derived from *BRCA2 999del5* mutation carriers and in four *BRCA2* heterozygous breast epithelial cell lines. The results suggest a role for BRCA2 in telomere maintenance by repressing telomere end-to-end fusions and telomere loss by replication stabilization.

The aims of this MSc-thesis are based on these and more results suggesting BRCA2 haploinsufficiency and a potential role for BRCA2 in telomere stabilization.

1 Introduction

Breast cancer is the most common cancer in women in the Western world (Kamangar et al. 2006). Despite advances in treatment and diagnosis, breast cancer remains to be the most common factor in cancer related deaths world-wide (Kamangar *et al*. 2006; Polyak 2007). In Iceland, breast cancer counts for about 30% of cancer in women with an average of over 200 diagnosis and about 40 deaths per year (years 2007-2011) and average age of onset about 60 year (Icelandic Cancer Registry, 2014). Early diagnosis and adequate treatment is essential for disease outcome. For the past 40 years, breast cancer 5 year survival rate in Iceland has risen from 55% in the years 1957-1966 to 90% in the years 1997-2006, presumably as a result of advances in treatment, public awareness and cancer screening resulting in earlier diagnosis (Jonasson *et al*. 2012). Investigation of the driving forces of tumor initiation and progression is far from being an easy task. Genetic analysis reveals that a single tumor is composed of wide range of tumor cells that have accumulated different mutations causing various proliferating effects growing along with normal cells and stroma (Hanahan *et al.*, 2011). Despite this heterogeneity of cells within a single tumor, molecular research and comparison between ethnic groups reveals that individual tumors are developing in distinct but certain pathways (Polyak, 2007). This intra- and intertumoral heterogeneity is different from one tumor to another so breast cancer is not a single disease but rather a group of different tumor types with different molecular properties and prognosis. The ranking of breast cancer to subgroups is important as aspects of cancer treatment can have different effects on disease outcome in each tumor type and is constantly changing as more knowledge accumulates.

Although the number of mutations and genetic alterations in tumors are high, key driver mutations in cancer development are in most cases believed to be relatively few or about 4-10 in each case. A range of hitchhiker mutations can follow the driver mutations that might have limited or no effect on tumor development (Vogelstein *et al*., 2004). These driver genes of tumor formation and development are divided into two categories that have an oppose function of each other. First, the tumor suppressor genes (TSG) that have a function of keeping cell functions intact and suppressing cell proliferation to the tissue specific normal rate. Second, the oncogenes have a function that can trigger increased proliferation in the cell and tumor formation (Hanahan *et al*., 2011). In a normal cell the two opposing functions of the TSG and oncogenes balance each other out keeping the cell intact at normal division rate for each type of tissue. Reduction of TSG function, i.e. changes in expression, allele loss or a mutation in the genes could lead to aberrations in cell cycle control and increased proliferating behavior compared to the normal surrounding tissue and might eventually lead to cancer properties. On the other hand, increased function of an oncogene, i.e. enhanced expression or allele copy-number changes, could trigger cell proliferation beyond the control of the TSG, leading to malignant outcome and tumor progression (Hanahan *et al*., 2011). Everyday environmental factors like mutagenic agents, ionizing radiation and free-radicals from cell metabolism can cause damage to the DNA threatening chromosomal integrity and genomic stability. Efficient pathways of DNA repair have evolved to maintain genomic stability and yet other pathways that force the cell to apoptosis or

senescence due to insufficient DNA repair. Some mutations, or composition of mutations, can bypass those mechanisms and eventually lead to tumor formation. Collective changes in six hallmarks have been proposed to be needed for cancer formation. Apoptosis resistance, evading growth suppression, sustaining proliferative signaling, enabling replicative immortality, inducing angiogenesis and activating invasion/metastasis (Hanahan *et al*., 2011). Additional factors that might play a role are the avoidance of immune system destruction, deregulation of cellular energetics and possibly tumor-promoting inflammation (Hanahan *et al*., 2011).

BRCA2 is a TSG that has an important role in DNA damage response mechanisms and homologous recombination (HR) repair of double strand breaks (DSB) (Venkitaraman 2002). BRCA2 has also been reported to have additional functions in the stabilization of stalled replication forks by preventing degradation of nascent DNA strands (Schlacher *et al*., 2011) and possibly in telomere maintenance (Badie *et al*., 2010; Bodvarsdottir et al. 2012 [Appendix paper 3]). Cancers derived from BRCA2 mutation carriers show abnormalities in chromosomal structure including tri- and quadriradials, broken chromosomes and chromatids suggesting an important function of BRCA2 in DNA maintenance and damage response (Gretarsdottir *et al*., 1998; Eyfjord & Bodvarsdottir 2005; Bodvarsdottir *et al*., 2012 [Appendix paper 3]).

1.1 BRCA2 and familial breast cancer.

In Iceland a germline founder mutation, the *BRCA2*^{999del5}, is responsible for 6-7% of all diagnosed breast cancers (Thorlacius *et al.,* 1996; Tryggvadottir *et al*., 2006). The initial step of tumor formation in *BRCA2* mutation carriers is yet not fully understood but is believed to be loss of the remaining wild-type allele. However, our studies and others have shown that *BRCA2* wild-type allele loss is not present in all tumors from *BRCA2* mutation carriers and might, in some cases at least, be a late event in tumor progression (King *et al*., 2007; Skoulidis *et al*.,2010 [Appendix paper 1]; Stefansson *et al*., 2011; [Appendix paper 2]; Aradottir *et al*., 2015).

Mutations in the *BRCA2* gene have been associated with predisposition to breast, ovarian, prostate and pancreatic cancers (Thorlacius *et al*., 1996; Venkitaraman *et al*., 2002). The Icelandic founder mutation, *BRCA2*^{999del5}, is a five base-pair deletion (nucleotides 999 to 1004), located in exon 9 of the *BRCA2* gene and is found in about 6-7% of all diagnosed female and 40% of male breast cancer cases (Thorlacius *et al*. 1997; Tulinius *et al*. 2002). Around 25% of breast cancer patients diagnosed by the age of 40 carry the *BRCA2* mutation and the incidence of developing breast cancer among carriers have shown to be around 70% plus a 4-5 fold risk of developing prostate or ovarian cancers (Thorlacius *et al*. 1997; Tryggvadottir *et al*., 2006; Tulinius *et al*., 2002). The *BRCA2*^{999del5} mutation leads to premature stop codon at nucleotide 1047 and a possible protein translation truncation. No mutant BRCA2 protein seems to be present in the cytoplasm (Taytigian *et al*., 1996; Michaelsdottir *et al*., 2004). Although no mutant BRCA2 protein is found in the cytoplasm, mutant mRNA transcript is produced so either the mutant mRNA transcript is less stable than the wild-type mRNA or the mutant BRCA2 protein is unstable and therefore not detected (Michaelsdottir *et al*.,

2004). Heterozygous *BRCA2* mutation carriers seem to show normal phenotype apart from their cancer predisposition (Welcsh *et al*., 2001). In *BRCA2*^{999del5} mutation carriers it has been shown that *BRCA2* prostate cancer patients have poor prognosis compared to non-carriers, lower age of onset (69 years versus 74 years), more advanced tumor stage and higher tumor grade at time of diagnosis and a lower median survival time (2.1 years versus 12.4 years in non-carriers) (Tryggvadottir *et al*., 2007). Recently, it was found that with average of 9.5 years follow up after diagnosis, a subgroup of BRCA2 breast cancer patients with a diploid phenotype showed significantly higher risk of death than non-carriers (Tryggvadottir *et al*., 2013). Other subgroups showing aneuploid phenotype had survival rate that was similar to non-carriers (Tryggvadottir *et al*., 2013).

1.2 Cancer and Knudsens' two hit hypothesis.

According to Knudsen's two hit hypothesis TSGs are haplosufficient i.e. only one allele is needed for gene function. Thus, TSG germline mutations seem always to be inherited in recessive manner and only inactivation of both alleles affects gene function and loss of cell cycle control and tumor formation. The hypothesis states that two "hits", one in each allele, are required for the loss of function of a specific TSG. When there is a genetic predisposition to cancer, like in *BRCA2* mutation carriers, the first hit is a single allele germline mutation found in every cell followed by a somatic second hit in the remaining wild-type allele. Thus, only one somatic mutation is required for tumor initiation explaining the increased cancer susceptibility to a certain extent. In non-hereditary tumors both the first and second TSG hits are somatic mutations. However, studies seem to indicate that this hypothesis might not be as definite as previously thought. Somatic tumors often present single allele mutations or a partial loss of TSGs (Berger *et al*., 2011; Stefansson *et al*., 2011 [Appendix paper 2]). Single allele mutations in genes of novel tumor suppressing function found in somatic cancers have therefore often been ruled out and explained as passenger mutations following another key driver mutation without effect on progression. However, there is a possibility that a single copy mutation in TSGs could have impact on cancer progression when accompanied by the lack of several cellular functions due to accumulated genetic alterations. It has been speculated that it might even be a selection for a single allele TSG loss in tumor progression as a selection pressure can arise favoring single allele loss in the abnormal state of the cancer cell genome whereas the homozygous TSG loss might be lethal or lead to apoptosis or senescence (Berger *et al*., 2011). Partial TSG loss could in those cases result in obligate haploinsufficiency and be more favorable for tumor development than a total TSG loss. Thus, the two hit model should be viewed to apply for TSG function, rather than for tumor initiation as additional mutations might be required for tumor progression.

Possible TSG haploinsufficiency should also be seen in this light. First, an obligate TSG haploinsufficiency might cause reduction or partial loss of specific cell function but only progress into tumor initiation when accompanied by an additional mutation that exposes the weakness from carrying only a single functional TSG allele (Berger *et al*., 2011). Second, a single allele germline mutation could yield a protein that interferes with the wild-type protein increasing the loss of function as well as

relieving selection pressure for a second hit (Arnold, 2006). Third possibility would be that the TSG is in fact haploinsufficient (Berger *et al*., 2011). In regard to *BRCA2* germline mutation, it is possible that carrying only a single wild-type *BRCA2* allele might play a role in tumor initiation without losing it, where in normal cells the dosage-sensitive loss of BRCA2 function might be masked by other genes and only exposed if those genes are mutated later on. This also leads to ideas of tissue specific haploinsufficiency in *BRCA2* mutation carriers as it remains to be explained why a germline mutation, in as globally important gene function as DNA repair, mostly leads to cancer in specific tissues such as breasts, ovaries and prostate glands.

1.3 DNA repair of double strand breaks

Several endogenous and exogenous factors pose a threat to cellular genome integrity during normal life of any given cell. Factors as replication errors, T- and B-cell development errors, ionizing radiation, radicals from cellular metabolism, ultra violet light and chemical agents can cause damage to the DNA backbone and lead to either single strand break or DSB in both DNA strands. DSB leaves a physical gap in the DNA strand and is one of the most severe form of DNA damage (Van Gent *et al*., 2001; Shiloh *et al*., 2004). As unrepaired DSBs can lead cells to senescence, apoptosis or tumor initiation, efficient cellular mechanisms have evolved to sense and deal with broken DNA strands as well as other DNA damage types. In eukaryotic cells two different pathways of repairing DSBs are known. First, the more error prone non-homologous end-joining (NHEJ) where any two loose DNA ends are joined together in a relatively straight forward manner and second, the more error free HR repair were the sister chromatid serves as a template for a more accurate repair (San Filippo *et al*., 2008; Lieber, 2010). The exact mechanism for selection of repair method is not yet well understood, however, it has been shown that HR is more likely to be involved at sites of replication fork collapse during S-phase and G2 phase of the cell cycle as it requires the sister chromatid as a template, were as NHEJ has been shown to function throughout the cell cycle (Brandsma *et al*., 2012). In NHEJ repair, the Ku complex (Ku70/80) is recruited at breakage site and coats the two broken DNA ends. Next step in the process is the recruitment of DNA-dependent protein kinase catalytic subunit (DNA-PKcs) at the site that phosphorylates itself among other targets in the NHEJ pathway such as nuclease protein Artemis that trims the DNA ends. The final stage is the recruitment of DNA ligase complex including XRCC4, DNA ligase IV and XLF that seals the break (Lieber, 2010).

In HR repair, the DSB is initially trimmed by MRN/X complex, consisting of MRE11 exonuclease, Rad50 and Nbs proteins, leaving a 3’ single stranded DNA (ssDNA) overhang. The ssDNA tails are covered with replication protein A (RPA) complex. More protein complexes are gathered and ssDNA tails are elongated further. RPA is replaced with RAD51 by BRCA2 and RAD51 paralogs among other proteins. The RAD51 and ssDNA nucleoprotein filament are capable of catalyzing invasion into homologous sister chromatid by the formation of a D-loop where the ssDNA end is elongated by DNA polymerases using the sister chromatid as a template. Two alternative

models of the final steps of HR repair have been proposed. First, the synthesis-dependent strand annealing where only one of the ssDNA strand is invaded into the D-loop and elongated resulting in non-crossover repair of the DSB. Second, DSB repair where both the 3' and 5' ssDNA of the initial DSB are captured in the D-loop, resulting in a double holliday junction that in the final step is cut and ligated. The DNA cleavage of the holliday junctions in DSB repair followed by gap-filling and DNA ligation can yield either crossover or non-crossover chromatid products. (San Filippo *et al*., 2008; Sung *et al*., 2006)

1.4 Fanconi anemia and DNA repair pathway of cross-linked DNA

Fanconi anemia (FA) is a rare autosomal or X-linked chromosomal instability disorder inherited in recessive manner. FA is clinically and genetically heterogeneous and diagnoses are complicated. FA patients show many developmental abnormalities like growth retardation, skeletal deformation, congenital anomalies and bone marrow failure as well as predisposition to cancer, mainly acute myeloid leukemia but also breast carcinoma and squamous cell carcinomas in the head and neck among others (Niedernhofer *et al*., 2005; Wang, 2007). Average life expectancy for FA patients is 22 years (Niedernhofer *et al*., 2005). To date, at least 15 different genes have been identified as likely the cause of FA, 14 of which are found on autosomal chromosomes and one on the X-chromosome, the *FANCB* (reviewed in Soulier, 2011). Many of the known FA proteins have enzymatic domains that are common in DNA interacting proteins such as those involving DNA-repair mechanisms. FA cells are DNA repair deficient and highly responsive to DNA-crosslinking agents like Mitomycin C (MMC) and Cisplatin (Niedernhofer *et al*., 2005). One of the FA-genes, the *FANCD1* has been found identical to *BRCA2*, and is in fact the same gene. Biallelic *BRCA2* (*FACND1*) loss is associated with FA-D1, a rare subtype of FA with distinct features from other FA-subgroups (Howlett *et al*., 2002; Offit *et al*., 2003). FA-D1 patients seem to show more predispositions to childhood onset of malignancies, predominantly medulloblastomas but also increased risk of in breast cancer and Wilm´s tumor (Offit *et al*., 2003).

The FA pathway is a response mechanism at stalled replication forks induced by crosslinks in the DNA and is activated by Ataxia telangiectasia and Checkpoint kinase 1 (Wang, 2007). The FA proteins are divided into three groups. First, the FA proteins in group-I are recruited at stalled replication forks and form a FA-core complex along with other proteins such as the Bloom protein complex (Wang, 2007). Second, after the core complex is formed, the crosslink is supposedly bypassed with transleision synthesis, believed to be activated by group-II FA proteins forming the ID-complex. After transleision, excision of the damaged (cross-linked) DNA is carried out by the nucleotide excision repair (NER) proteins, leaving a DSB in the nascent DNA-strand. Third, the remaining DSB is repaired by group-III FA-proteins, using HR process to complete the FA-repair pathway so DNA synthesis can continue as normal. The HR repair proteins involve BRCA2/FANCD1

among others (Wang, 2007). Thus, the BRCA2/FANCD1 protein (Group-III) is working downstream of the main FA-core complex (Group-I) and the ID complex (Group-II).

Lymphocytes in FA patients seem to show similar alterations in chromosome structure as found in breast cancers of *BRCA2* carriers, including end-to-end fusion and radial chromosome formation, as discussed further in the following chapter (Eyfjord and Bodvarsdottir, 2005; Bodvarsdottir *et al*., 2012; [Appendix paper 3])

1.5 Chromosome alterations in breast cancer

Breast cancers can be classified into subgroups by their molecular profile and phenotype. Biomarkers such as progesterone receptor (PR), human epidermal growth factor receptor 2 (HER2) and estrogen receptor (ER), among others, are commonly used for molecular and clinical analysis (Perou *et al*., 2000; Chin *et al*., 2006). Gene expression profiling has led to the categorization of breast cancer into five different subgroups: Luminal A, Luminal B, HER2 positive, normal like and basal like (Polyak, 2007). Luminal A and B are ER and/or PR-positive but Luminal B is HER2-postitive and Luminal A is not. HER2 positive tumors are ER and PR negative (Polyak, 2007; Cheang *et al*., 2008). Basal-like tumors most frequently show a triple negative phenotype i.e. ER, PR and HER2 negative. Comparative genomic hybridization (CGH) profiling on genomic alterations in BRCA tumors reveal that *BRCA1* mutated breast tumors frequently categorizes as basal-like whereas *BRCA2* mutated breast tumors most frequently are of luminal subtypes, suggesting that mutations in the two *BRCA* genes lead to different pathways of tumor formation (Stefansson *et al.*, 2009). Multiple chromosomal alterations and rearrangements are characteristic for *BRCA2* mutated breast tumors such as chromatid breaks, chromosome end-to-end fusions (CEFs), radial chromosome formation as well as telomere abnormalities (Tirkkonen *et al*.,1997; Gretarsdottir *et al*., 1998; Bodvarsdottir *et al*., 2012 [Appendix paper 3]). This may result from deficiencies in different roles of BRCA2 like repair of DSB via HR, replication fork collapse but also suggest a possible role for BRCA2 in telomere maintenance. It has also been shown that BRCA2 seems to have a role in cytokinesis and that dysfunctions in the cell cycle, such as breakage-fusion-bridge (BFB) cycles and anaphase bridges can lead to abnormalities in chromosome number and structure (Eyfjord and Bodvarsdottir, 2005; Jonsdottir *et al*. 2009).

1.6 BRCA2 and loss of heterozygosity

Loss of the wild-type *BRCA2* allele in breast tissue of a *BRCA2* mutation carrier was presumably thought to be the initial step of development of breast cancer. Our studies have shown that only about 50% of breast, prostate and pancreatic cancers, derived from *BRCA2*^{999del5} mutation carriers, have lost their wild-type *BRCA2* allele in more than 50% of the cells (Bjarnason, 2009; Skodulidis *et al*., 2010

[Appendix paper 1] Stefansson *et al*., 2011 [Appendix paper 2]; Aradottir et al., 2015). In our study of seven pancreatic tumors from patients inheriting *BRCA2*^{999del5}, three out of four pancreatic ductal adenocarcinomas (PDACs) did not exhibit loss-of-heterozygosity (LOH) of the *BRCA2* wild-type allele (Figure 1). All three acinar carsonomas from these patients, however, displayed *BRCA2* LOH which also developed only in mice with biallelic *Brca2* inactivation in the study (Skodulidis *et al*., 2010 [Appendix paper 1]). Interestingly, breast cancer analysis revealed that the proportional wild-type to mutant allele composition in the tumors showed a continuous range from total wild-type allele loss to no detectable loss and that the wild-type allele loss is correlated with Ki67 expression, a strong cell proliferation marker, measured in the same breast tumors (Stefansson *et al.* 2011 [Appendix paper 2]). Although when no or minor *BRCA2* wild-type allele loss was detected, some of these breast tumors or cell lines still showed chromosomal aberrations suggesting haploinsufficient effect of BRCA2 (Bodvarsdottir *et al*., 2012 [Appendix paper 3]). Hence, there is a question whether tumor initiation in *BRCA2* carriers might, in some cases at least, be due to other factors than *BRCA2* wild-type allele loss making it a potential late event in tumor progression.

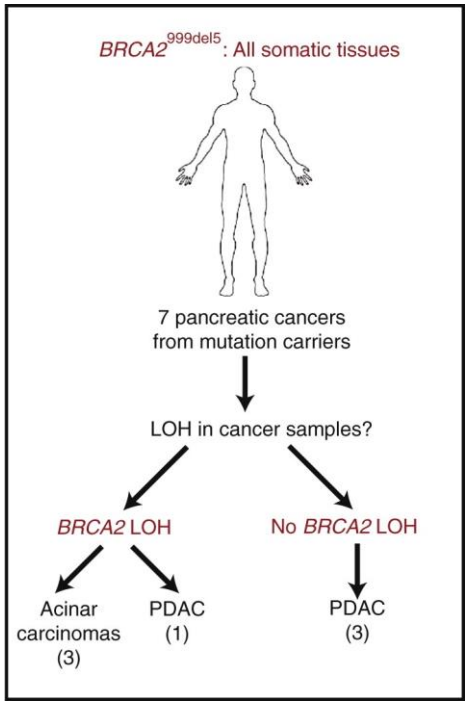

Figure 1. About half of the pancreatic cancers from *BRCA2*^{999del5} mutation carriers had lost their wild-type *BRCA2* allele by loss of heterozygosity (LOH). Those tumors that were with *BRCA2* LOH had developed to acinar carcinomas, except one, and those that did not have *BRCA2* LOH were of pancreatic ductal adenocarcinoma (PDAC) origin (Modified from Skodulidis *et al*., 2010 [Appendix paper 1]).

1.7 BRCA2 and telomeres

Telomeres are DNA-protein structures on chromosome ends composed of tandem repeats of $(TTAGGG)_n$ sequences, about 10-15 kb long in humans. The TTAGGG repeats associates with six proteins that are called shelterins. TRF1, TRF2 and POT1 bind directly to the telomere sequences and together with TIN2, TPP1 and Rap1 they form the shelterin complex. Telomere repeats and shelterin proteins form a structure where the 3' overhang (about 150-300kb long) is integrated into a D-loop in

the double stranded part of the telomeric region to form a so called T-loop (de Lange, 2005). This higher order structure serves to protect the chromosome ends from being handled and repaired as a common DSB and that way maintains chromosomal structure and integrity (de Lange, 2005).

Research on *BRCA2* mutations have revealed a connection to gross chromosomal changes through a defective HR repair of DSB that can lead to genomic instability, cancer initiation and progression (Moynahan *et al*., 2001). In recent years, indications on a role for BRCA2 in telomere stabilization have emerged. We showed that breast tumors from *BRCA2* mutation carriers showed a higher frequency of CEFs, an indication of dysfunctional telomeres, than in non-carriers (Bodvarsdottir *et al* 2012 [Appendix paper 3]). The study also showed that in breast epithelial cell lines, carrying the same *BRCA2* mutation, CEFs were also found to be more frequent compared to wild-type control as well as showing other factors indicating BRCA2-linked telomere defects like interstitial telomere sequences (ITS), telomere sister chromatid exchanges (T-SCEs), extrachromosomal telomeric repeats (ECTR) and telomere dysfunction-induced foci (TIFs) formation (Bodvarsdottir *et al* 2012[Appendix paper 3]). Another study found an increase in CEFs indicating capping defects, single telomere signals indicating loss of telomeric repeats, TIFs and T-SCE following a cre-loxP depletion of BRCA2 in proliferating mouse embryonic fibroblasts (MEFs) (Min *et al*.,2012). The role of BRCA2 in telomere maintenance seems to be complex. As T-SCE is a key function in cells using alternative lengthening of telomeres (ALT) a study was conducted using *BRCA2* siRNA knockdown method to compare T-SCE response in ALT-positive and ALT-negative fibroblast cell lines. Results showed two features of BRCA2 impact on telomere recombination. BRCA2 presence seems to suppress T-SCE in ALT-negative cells, whereas BRCA2 depletion reduced levels of T-SCE normally found in ALT-positive cell lines (Sapir *et al*., 2011). BRCA2 has been shown to load RAD51 recombinase onto telomeres during cell proliferation, contributing to telomere integrity (Badie *et al*., 2010). BRCA2 depletion and RAD51 inhibition in MEFs led to fragmented telomere signals and telomere shortening suggesting that BRCA2 facilitates telomere replication and maintenance through HR mediated reactions in normal cell proliferation (Badie *et al*., 2010). Genomic instability seen in *BRCA2* tumors might therefore partly be due to telomere dysfunction as well as HR deficiency.

2 General aims

The first aim of the project was to examine how cells respond to the DNA mutagenic substance MMC in regard to *BRCA2* status. The fact that a proportion of tumors derived from *BRCA2* germline mutation carriers seems to show low or no detectable loss of the *BRCA2* wild-type allele gives rise to the question whether a mild haploinsufficiency of the *BRCA2* gene might affect tumor formation in these individuals. The drug used, MMC, causes DNA cross-links and is one of the drugs used in clinical testing for FA were cells show reduced DNA repair capacity. *BRCA2* (or *FANCD1*) is one of several genes known to be responsible for FA. FA-cells show an increase in the formation of chromosome breaks and different chromosomal abnormalities such as radial- and dicentric chromosome formation when treated with a DNA mutagenic substance. Thus, this project utilizes these abnormalities as parameters for addressing the question whether haploinsufficiency can be

measured in *BRCA2* heterozygous cells by comparing chromosomal aberrations in three different *BRCA2* genotypes; the *BRCA2* wild-type (+/+), *BRCA2* heterozygous mutant (+/-) and *BRCA2* homozygous mutant (-/-) cells. Chromosomal analysis were done on conventional solid Leismans' stained metaphase spreads using a bright field microscope.

The second aim of this study was to follow up on our findings of *BRCA2* related telomere dysfunction (Bodvarsdottir *et al*.,2012 [Appendix paper 3]) when treated with MMC. Telomere dysfunctional parameters such as single telomere signals, telomere free ends, CEF, MTS and ITS were analyzed as potential indicators of dysfunction in DNA repair and telomere replication errors in the three different *BRCA2* genotypes.

Specific aims were to:

- Analyze differences in chromosomal stability between cells with different *BRCA2* genotypes
- Analyze differences in efficiency of DNA repair between *BRCA2* genotypes after treatment with a crosslinking agent
- Find out if *BRCA2* haploinsufficiency affect DNA repair, chromosomal stability or telomere maintenance
- Analyze if telomere replication and maintenance is affected by different *BRCA2* genotypes

3 Material and methods

3.1 Lymphocyte cell lines

Eleven non-commercial lymphocyte cell lines were used in this study, ten of which were transformed in our laboratory and have not been reported before (table 1). Three lymphocyte cell lines were derived from individuals with wild-type *BRCA1/2* (EB1117, EB0392, EB6454). Five *BRCA2*-heterozygous cell lines were from carriers of the $BRCA2^{999del5}$ mutation (EB1482, EB1690, EB1830, EB2302 and EB6085). One *BRCA2*-homozygous cell line derived from a FA-D1 patient carrying the (probably) deleterious IVS19-1G→A mutation in both alleles (HSC62N) (Howlett *et al*., 2002). The HSC62 cell line is lacking 12 base-pairs in exon 20 leading to an in-frame 4 amino-acid deletion. HSC62 cells produce a normal sized BRCA2 protein that still might have partial activity which correlates with the fact that the patient of which the cell line was derived from showed a clinically mild FA-syndrome (Howlett *et al*., 2002; Alter, 2007). The HSC62N cell line was kindly provided from Dr. Helmut Hanenberg at the Herman B. Wells Center for Pediatric Research at Indiana University. Two *BRCA1*-heterozygous cell lines were derived from *BRCA1* 5193G->A mutation carriers (EB6420 and EB6454). Cell lines, *BRCA*-status and culture medium are listed in table 1. All cell lines were cultured in 50mL polystyrene tissue culturing flasks (Becton Dickinson Labware, Franklin Lakes, NJ, USA) and maintained in RPMI 1640 + 10% FBS culture medium at 37°C in a humidified 5% CO_2 atmosphere with added penicillin (50 IU/ml) and streptomycin (50 µg/ml) (GIBCO) (Table 1), except for HSC62N

where non heat-inactivated FBS had to be used for cell growth. FBS was purchased from GIBCO and heat-inactivated at 56°C for 30 minutes and filtered before use except in the case of HSC62N.

Table 1. Lymphocyte cell lines. Cell origin, transformation method, *BRCA* mutation status and culture medium of each lymphocyte cell lines used for chromosomal aberration and telomere dysfunction analysis are listed.

Cell line	Cell type	Transformation	*BRCA* status	Culture medium
EB1117	Lymphocyte	EBV	*BRCA1/2*$^{+/+}$	RPMI 1640 + 10% FBS
EB0392	Lymphocyte	EBV	*BRCA1/2*$^{+/+}$	RPMI 1640 + 10% FBS
EB6457	Lymphocyte	EBV	*BRCA1/2*$^{+/+}$	RPMI 1640 + 10% FBS
EB1482	Lymphocyte	EBV	*BRCA2*$^{+/-}$	RPMI 1640 + 10% FBS
EB1690	Lymphocyte	EBV	*BRCA2*$^{+/-}$	RPMI 1640 + 10% FBS
EB1830	Lymphocyte	EBV	*BRCA2*$^{+/-}$	RPMI 1640 + 10% FBS
EB2302	Lymphocyte	EBV	*BRCA2*$^{+/-}$	RPMI 1640 + 10% FBS
EB6085	Lymphocyte	EBV	*BRCA2*$^{+/-}$	RPMI 1640 + 10% FBS
HSC62N	Lymphocyte	EBV	*BRCA2*$^{-/-}$	RPMI 1640 + 10% non-heat inactivated FBS
EB6420	Lymphocyte	EBV	*BRCA1*$^{+/-}$	RPMI 1640 + 10% FBS
EB6454	Lymphocyte	EBV	*BRCA1*$^{+/-}$	RPMI 1640 + 10% FBS

3.2 Mitomycin C treatment

MMC ($C_{15}H_{18}N_4O_5$, figure 2; obtained from Sigma-Chemical Company St.Louis MO, USA; lot 89F-0016) was dissolved in distilled water and stock solution made (500 µg/ml). MMC is a natural product isolated from *Streptomyces caespitosus* and is used as a chemotherapeutic agent for its antitumor antibiotic activity serving as a potent DNA cross-linker. According to manufacturer, MMC has a half-life of approximately 60 minutes in cell culture medium at 38°C, supplemented with antibiotics and fetal calf serum. In all experimental setups cells were treated with 50 ng/ml of MMC and cultivated for 72 hours without changing medium followed by chromosome harvest. As 50 ng/ml is a relatively low dosage and the lifespan of active MMC is short, the medium was not exchanged after drug inducement and the 3 day follow-up cultivation mostly considered as a recovery period.

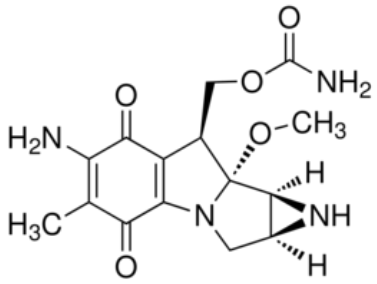

Figure 2. Chemical structure of Mitomycin C.

3.3 Chromosome harvest and metaphase spreads

For metaphase arrest 8 µl colcemid (stock concentration of10µg/ml; GIBCO, KaryoMAX–colcemid, Lot.879518) was added to each milliliter of culture medium. Cell cultures were incubated for approximately three to four hours prior to chromosome harvest. Cell cultures were then centrifuged in sterile 15 ml centrifuge tubes (BD Falcon, REF. 352096) in the culture medium for 10 min at 325-rcf. Supernantant was removed and pellet mixed using siliconized glass-pipette in 7 ml of 0,075 M KCl at 37°C and incubated for 10 minutes at room temperature. One milliliter of freshly made fixative (3 parts-absolute methanol / 1 part-100% glacial acetic acid) was added to each tube. Centrifuged for 10 min at approximately 325-rcf. Supernatant removed carefully down to white buffy layer. Pellet was mixed slowly with fresh fixative on vortex, added drop by drop at first with gradually increasing pace, to an end volume of 4 ml and centrifuged for 10 min at approx. 325-rcf. Supernatant was removed, pellet mixed with fixative on vortex to end-volume of 3 ml and centrifuged for 10 min at 325-rcf. Pellet was mixed with small amount of fixative (until solution looked like whey) and stored at -20°C.

Metaphase spreads were made by releasing a drop of fixed cells from siliconized glass pipette approximately 5-10 cm above onto chilled and absolute ethanol wet microscope slide that had been stored at -20°C. Variation in chromosome spreading was controlled by adjusting humidity during droplet drying placing slides on dry or wet paper towels or over a bowl of steaming hot water during the drying period of the droplet. Higher humidity in surroundings results in longer drying period and more scattering of chromosomes.

3.4 Chromosome abnormality analysis

Metaphase spreads were prepared by solid Leishman's-staining (eosin-polychrome methylene blue) for 3 minutes using 1 part Leishman's stain (SIGMA, L-6254, EEC no. 235-732-1) 3 parts pH 7,2 phosphatebuffer. Photos were shot using Leica DM LB bright-field microscope and 100x oil-lens and 0.33-1.60x C-mount adapter (Leica 541 517 HC) using Leica DCF310 FX camera. Chromosomal abnormalities analyzed were double strand breaks, CEFs on one or both chromatids (dicentric chromosomes), radial chromosome formation and DNA fragments (Figures 3 A-C).

Abnormalities were analyzed in untreated cells and MMC treated (50 ng/ml) cells followed by 72 hours chromosome repair period and calculated per metaphase. The goal was to score around 80 metaphases in each culture that ended up in range of 60-83, except for EB1830 (MMC treated) only 21 metaphase was scored and for EB1690 (MMC treated) only 32 metaphases were scored, due to low survival rate after treatment with 50 ng/ml MMC (Table 2). Results from treated and untreated cell lines are presented with standard error of mean.

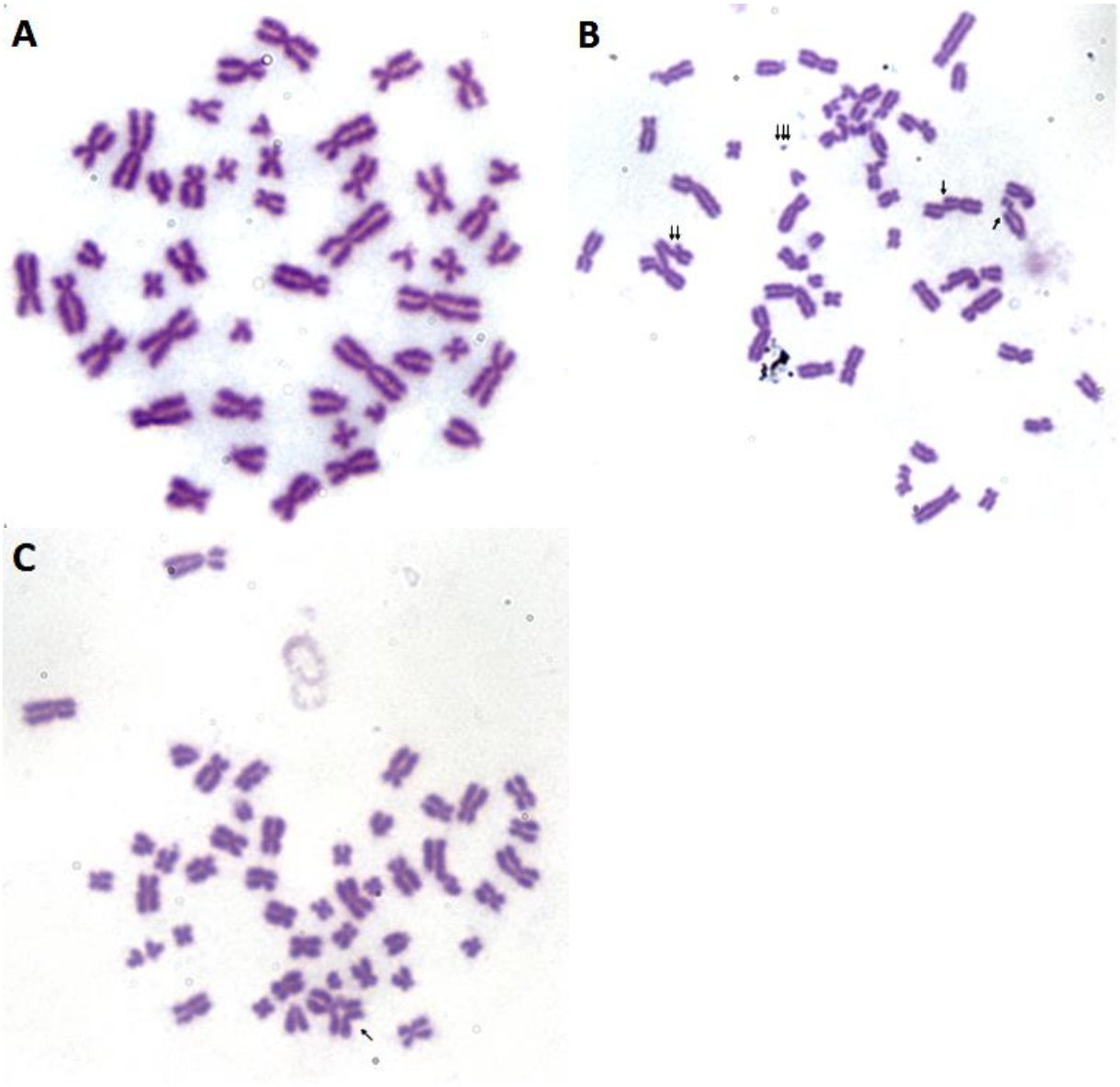

Figure 3. Metaphase spreads with Leishman's stain. **A**. Lymphocyte control cell line EB392 without MMC treatment showing a normal karyotype and no chromosomal abnormalities. **B**. Fanconi D1 lymphocyte HSC62N cell line after MMC treatment showing double strand breaks,(single arrow), chromatid end-to-end fusion (double arrows) and a DNA fragment (triple arrows). **C** Fanconi D1 lymphocyte HSC62N cell line after MMC treatment showing radial formation (single arrow) among other abnormalities.

3.5 Telomere FISH analysis

Fluorescence *in situ* hybridization (FISH) is a technique where sequence specific probes are used for analyzing the presence, absence or localization of specific DNA or RNA targets. The fluorescence labelled probes are hybridized to the targets and an epifluorescence microscope used to excite the probes by illuminating the specimen with the probe specific excitation wavelength. The probe then emits light of a different wavelength, the emission wavelength.

Peptide nucleic acid (PNA) probes are synthetized polymers that resemble DNA or RNA probes but has N-glycine as back bone with purines and pyrimidines attached and have stronger binding to DNA and RNA than regular DNA probes.For telomere dysfunction analyzes, ready-to-use telomere PNA FISH kit / Cy3 (K5326 from Dako) was used along with pan-centromeric FITC conjugated PNA probe for *in situ* hybridization of all telomeres and centromeres. Probe hybridization: 4 µl of PNA-centromere-green and 4 µl all-telomere-red PNA-probe in hybridization buffer were mixed and added on metaphase slides. 18 x 18 mm cover glass was glued on top of the slides followed by denaturation at 80°C for 5 minutes on a hybridizer (DAKO) and then 1 hour hybridization incubation period in a humid box at room temperature. Post hybridization stringency wash was performed to wash off unhybridized and excess probes by removing cover glass and washing slides twice for 10 minutes in 2 x SSC (saline-sodium citrate) buffer + 0,1 % soap (Tween 20) at 60°C. Slides were then rinsed with diluted water and air dried before mounting with DAPI stained antifade and topped with a 24 x 60 mm cover glass. Metaphases were photographed in Leica epifluorescent microscope using 100 x oil-lens and 0.33 - 1.60 C-mount adapter, either directly after hybridization or after overnight storage at 4°C.

Photos of metaphases after telomere FISH were analyzed for telomere dysfunctions like single telomere signals on chromatid ends, telomere free chromosome ends, multi telomere signals and ITS in both untreated and MMC treated (50 ng/ml) cells followed by 72 hours chromosome repair period (Figure 4). Around 70 metaphases were scored from each cell line and abnormalities were calculated per metaphase. Results from treated and untreated cell lines are shown with standard error of mean.

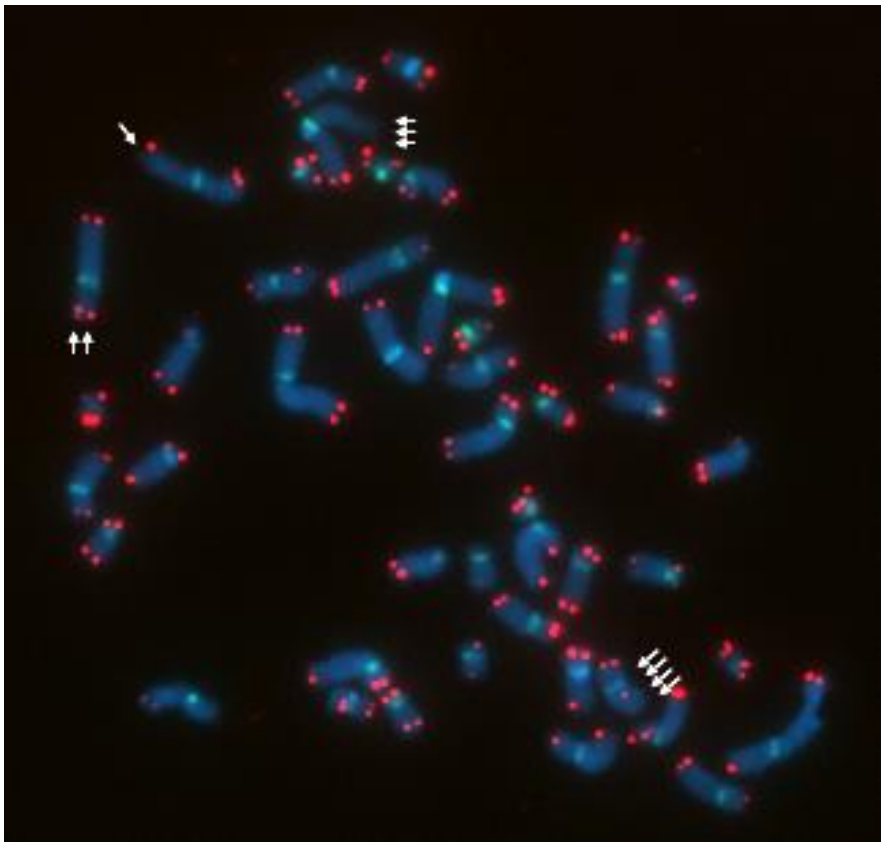

Figure 4. Metaphase spread with FISH stain in lymphocyte cell line EB2302 without MMC treatment. The telomere specific red PNA-probe binds to all telomere sequences and centromere specific green probe binds to centromeres on all chromosomes, DAPI blue binds to all DNA. Dysfunctions analyzed were single telomere signals (single arrow), multi telomere signal (double arrow), telomere-free end (triple arrow) and interstitial telomere sequence (quadruple arrow).

3.5.1 Statistical analyzes

All abnormalities were scored as per metaphase. Results are presented as an average mean with SEM bars. As for *BRCA2* status comparison, data of cell lines sharing the same genotype were pooled and an average mean calculated and presented with SEM bars. Statistical significance was calculated using two tailed unpaired t-tests. Significance is indicated as stars as following; ns $P > 0.05$, * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$

4 Results

4.1 Chromosomal abnormalities in lymphocyte cell lines

Metaphase spreads from eleven lymphocyte cell lines with and without 50 ng/ml MMC treatment and 72 hour follow up culture were analyzed for chromosomal abnormalities. Three wild-type (*BRCA2*$^{+/+}$) cell lines, five heterozygous (*BRCA2*$^{+/-}$) cell lines carrying the hereditary *BRCA2 999del5* mutation, one homozygous (*BRCA2*$^{-/-}$) cell line, the HSC62 carrying the *IVS19-1G→A* mutation in both alleles, and two heterozygous *BRCA1* cell lines carrying the hereditary *5193G-> A* mutation were analyzed for breaks, CEF's, radial formations and fragments (Figures 5 A-D; Table 2).

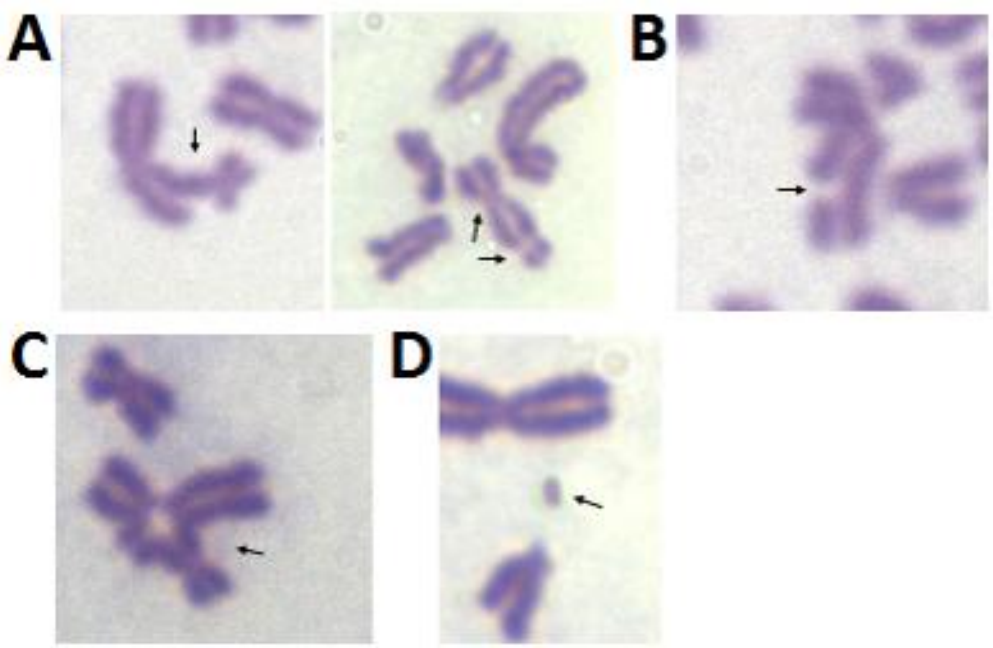

Figure 5. Chromosomal abnormalities analyzed. **A**. End-to-end fusion on single chromatids in cell line HSC62N after MMC treatment (left) and end-to-end fusions on both chromatids (dicentric chromosome) in cell line EB6454 after MMC treatment (right). **B**. Double strand break on a single chromatid in cell line HSC62N after MMC treatment. **C**. Radial chromosome formation in HSC62N after MMC treatment. **D**. DNA fragment in cell line EB6454 after MMC treatment.

Table 2: Lymphocyte cell lines analyzed for chromosomal abnormalities with and without MMC treatment. Values are average numbers per metaphase.

			Untreated - avarage per metaphase					MMC treated - avarage per metaphase			
Cell line	Genotype	Metaphases analyzed Untreated	Double strand breaks	End fusions	Radial aberrations	DNA fragments	Metaphases analyzed MMC treated	Double strand breaks	End fusions	Radial aberrations	DNA fragments
EB1117	BRCA1/2+/+	65	0,169	0,0153	0	0	65	0,585	0,6	0,215	0,0308
EB0392	BRCA1/2+/+	80	0,0125	0	0	0	79	0,304	0,215	0,114	0,203
EB6457	BRCA1/2+/+	83	0,0482	0,0361	0	0	78	0,962	0,538	0,128	0,244
EB1482	BRCA2+/-	81	0,0123	0,0123	0	0,0247	66	1,242	0,924	0,273	0,0909
EB1690	BRCA2+/-	73	0,0137	0,0548	0	0	32	0,563	0,906	0,0938	0
EB1830	BRCA2+/-	81	0	0,0494	0	0	21	1,0476	0,952	0,286	0,0476
EB2302	BRCA2+/-	81	0,0617	0,0494	0	0,0247	80	0,363	0,2	0,0625	0,0625
EB6085	BRCA2+/-	78	0,0513	0,0385	0	0	76	0,224	0,329	0,0395	0,0921
HSC62N	BRCA2-/-	70	0,414	0,1	0,0714	0,0286	60	3,217	1,75	0,217	0,217
EB6420	BRCA1+/-	78	0,0128	0,0128	0,0128	0	79	0,266	0,241	0,114	0,0253
EB6454	BRCA1+/-	81	0,0123	0	0,0123	0	84	1	0,537	0,232	0,256

All *BRCA2* wild-type lymphocyte cell lines showed statistically significant difference in chromosomal abnormalities due to crosslinking effect from treatment with 50 ng/mL MMC (Figure 6). Chromosomal abnormalities analyzes in cell line EB1117 showed 3.4 fold increase in break formation and 40 fold increase in chromosome CEFs when treated with MMC (Figure 6A). Radial formation and DNA fragments appeared as well but were not detected in the untreated sample. Cell line EB6457 showed 20 fold increases in breaks and 15 fold increases in CEFs. Radial formations appeared as well but were not detected in untreated sample (figure 6B). Lymphocyte cell line EB392 showed 24 fold increases in breaks after MMC treatment. CEFs, radial aberrations and fragments also appeared but were not detected when untreated (Figure 6C).

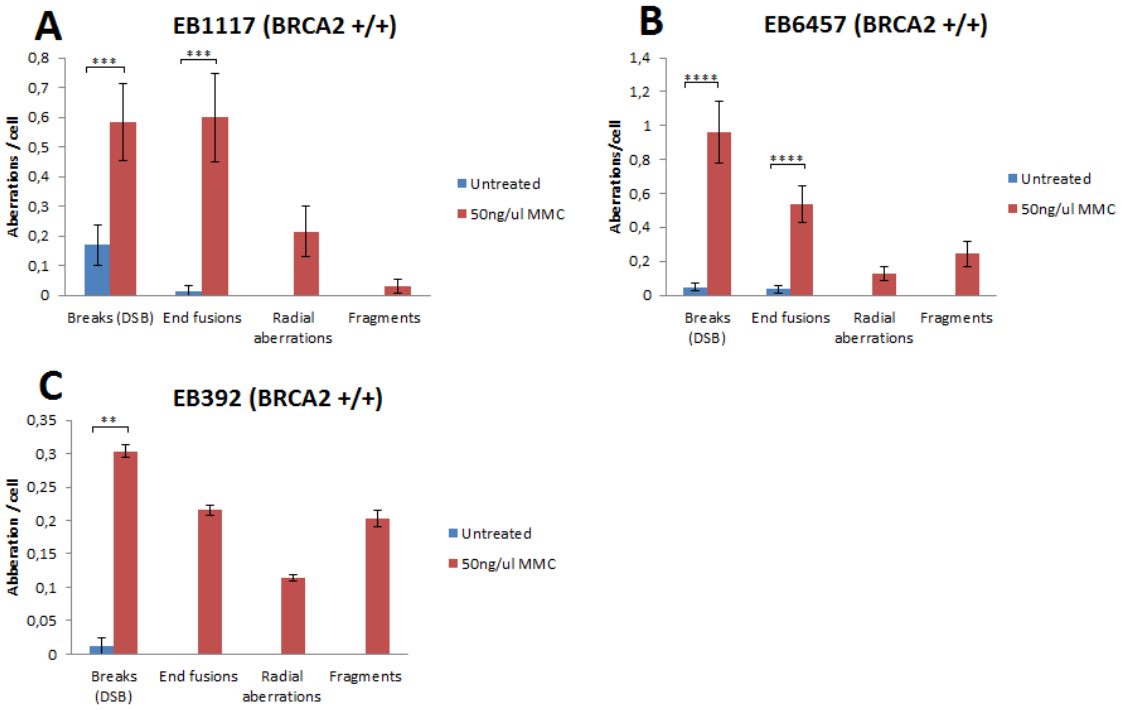

Figure 6. Chromosomal abnormalities in three *BRCA2* wild-type (*BRCA2*$^{+/+}$) cell lines. Blue columns show untreated samples and red columns show abnormalities when treated with 50 ng/ml MMC. Cell lines **A**) EB1117 (*BRCA2*$^{+/+}$), **B**) EB6457 (*BRCA2*$^{+/+}$) and **C**) EB392 (*BRCA2*$^{+/+}$). All cell lines had significant increase in chromatid breaks and two of three cell lines had significant increase in chromosome end-to-end fusions. Radial configurations and fragments were only detected after MMC treatment. ns $P>0.05$, * $P\leq 0.05$, ** $P \leq 0.01$, *** $P\leq 0.001$, **** $P \leq 0.0001$

All *BRCA2* heterozygous cell lines showed statistically significant increase in chromosomal abnormalities due to MMC treatment (Figure 7). Lymphocyte cell line EB1482 showed 103 fold increase in breaks, 77-fold increase in CEFs and 3.6 fold increase in fragments (Figure 7A). Radial chromosomes were not detected in the untreated sample but appeared as well after MMC treatment. EB1830 showed an increase in CEFs of 19.4 fold and breaks, radial- and fragment formation also

appeared (Figure 7B). In cell line EB6085 breaks showed 4.3 fold increase and 8.7 fold increase in CEFs (Figure 7C). Radial formations and fragments were only detected in the treated sample (Figure 7C). EB1690 showed chromatid break increase of 41 fold and 16 fold increase in CEFs. Fragments and radial formation were only detected in treated samples (Figure 7D). Cell line EB2302 showed 6 fold increase in breaks, 4 fold increase in CEFs and 2.4 fold increase in number of fragments when treated with MMC. Radial formation was not detected in untreated sample (Figure 7E).

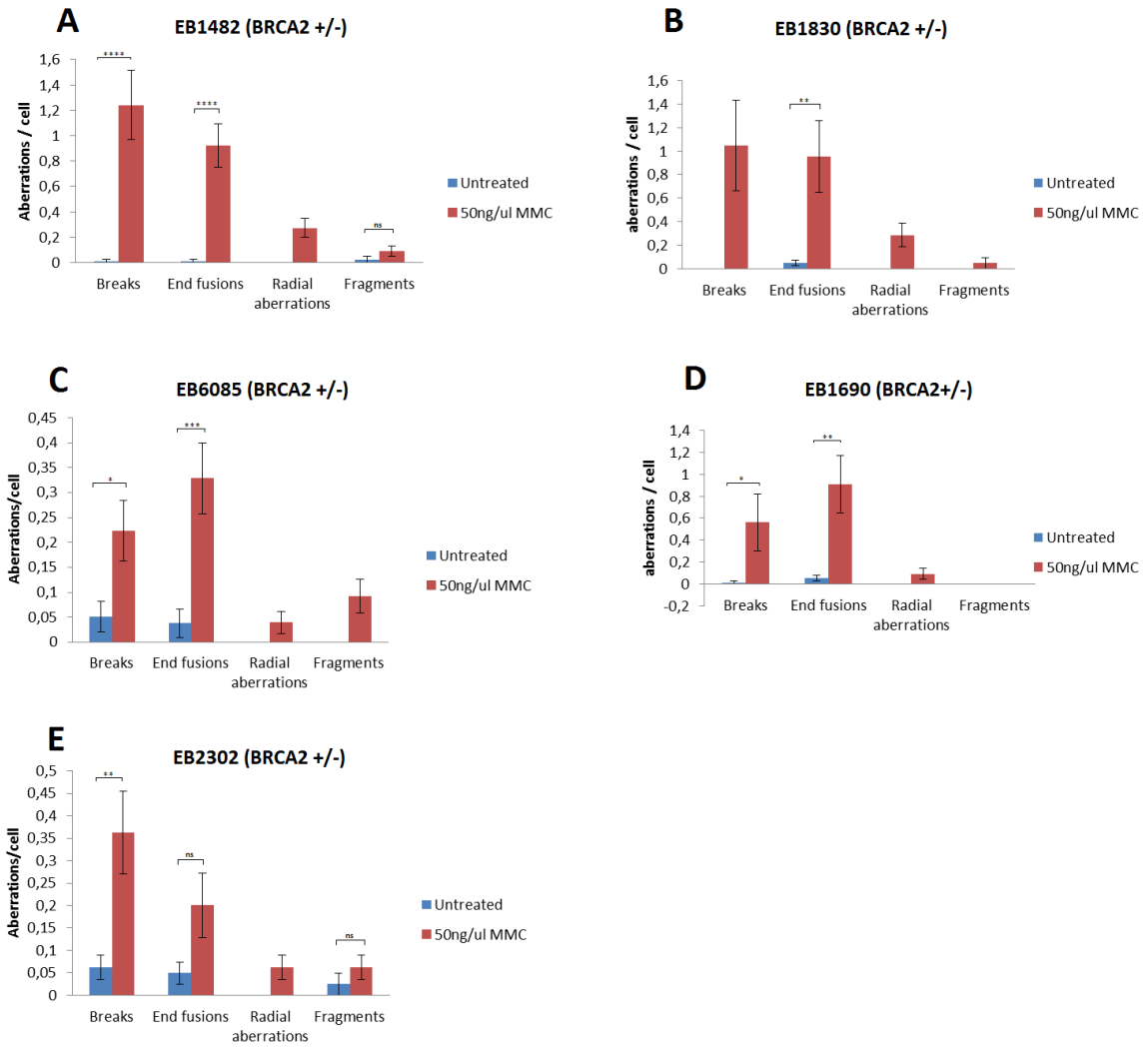

Figure 7. Chromosomal abnormalities in five *BRCA2*-heterozygous (*BRCA2*$^{+/-}$) cell lines. Blue columns show results from untreated samples and red show abnormalities when treated with 50 ng/ml MMC. Cell lines **A**) EB1482 (*BRCA2*$^{+/-}$), **B**) EB1830 (*BRCA2*$^{+/-}$), **C**) EB6085 (*BRCA2*$^{+/-}$), **D)** EB1690 (*BRCA2*$^{+/-}$), and **E)** EB2302 (*BRCA2*$^{+/-}$). All cell lines showed increase in chromatid breaks and end-to-end fusions after MMC treatment. Two cell lines also showed increase in chromosome fragments, but most of the cell lines did not show any radial configurations nor chromosome fragments when not treated with MMC. ns P>0.05 , * P≤ 0.05, ** P ≤ 0.01, *** P≤ 0.001, **** P ≤ 0.0001.

The *BRCA2* homozygous cell line HSC62N (FA-D1) showed a statistically significant 7.7 fold increase in chromatid breaks, 17 fold increase in CEFs and 7.6 fold increase in chromosome fragments after MMC treatment (Figure 8). Radial chromosome formation increased 3 fold, although not statistically significant (Figure 8). The FA cell line HSC62N was the only cell line analyzed that showed defects in all of the four chromosomal alteration categories untreated.

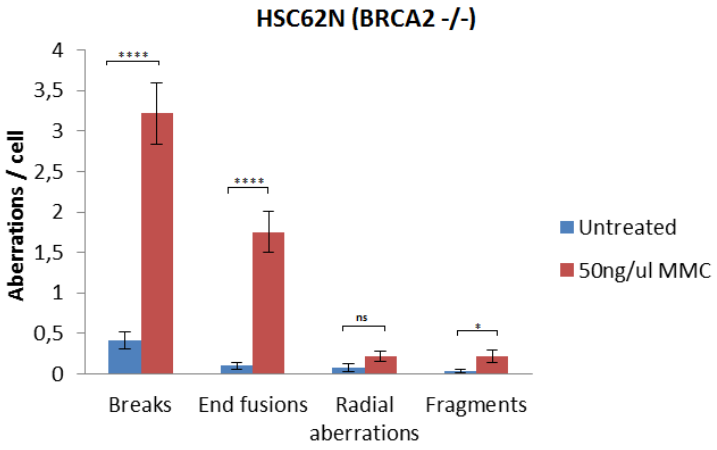

Figure 8. Chromosomal abnormalities in the *BRCA2*-homozygous (*BRCA2*$^{-/-}$) cell line HSC62 (Fanconi anemia-D1). Blue columns show untreated samples and red show abnormalities when treated with 50 ng/ml MMC. Differences in chromosomal changes between untreated and MMC treated cell lines were significant for all subgroups. ns $P>0.05$, * $P\leq 0.05$, ** $P \leq 0.01$, *** $P\leq 0.001$, **** $P \leq 0.0001$

Both *BRCA1* heterozygous cell lines showed statistically significant increase in chromosomal abnormalities analyzed when treated with 50 ng/ml MMC. The lymphocyte cell line EB6454 showed 83 fold increase in breaks and 19.2 fold increase in radial formation after MMC treatment (Figure 9A). CEFs and DNA fragments appeared with MMC treatment but were not detected in the untreated sample. Cell line EB6420 showed 20.5 fold increase in chromatid breaks, 18.5 fold increase in CEFs and 8.8 fold increase in radial formation after MMC treatment (Figure 9B). DNA fragments appeared due to MMC treatment, but were not detected in untreated samples.

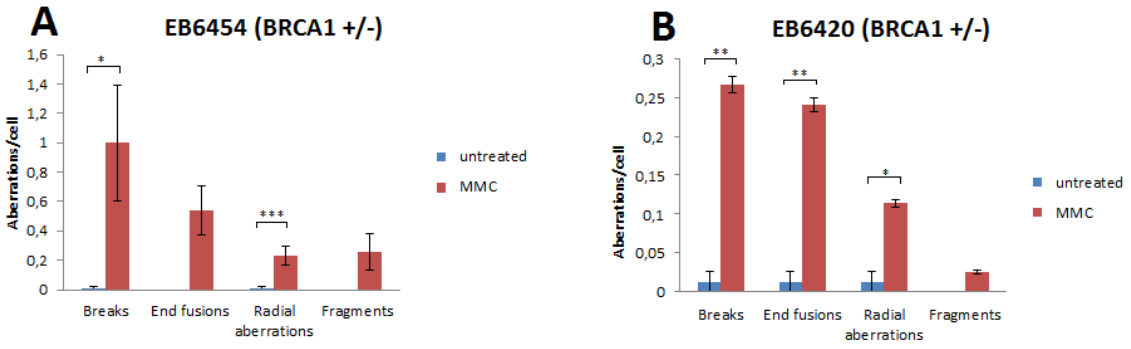

Figure 9. Chromosomal abnormalities in the two *BRCA1* heterozygous (*BRCA1*$^{+/-}$) cell lines. Blue columns show untreated samples and red show abnormalities when treated with 50ng/ml MMC. Cell lines **A)** EB6454 (*BRCA1*$^{+/-}$) and **B)** EB6420 (*BRCA1*$^{+/-}$). Both cell lines had significantly more chromatid breaks and radial configurations after MMC treatment. One of the cell lines also had significant increase in end-to-end fusions. No chromosome fragments were detected in the cell lines before MMC treatment. ns P>0.05 , * P≤ 0.05, ** P ≤ 0.01, *** P≤ 0.001, **** P ≤ 0.0001

4.1.1 Chromosomal abnormalities in different *BRCA2* genotypes without MMC treatment

For the three *BRCA2*$^{+/+}$ cell lines a total of 228 metaphases (65 to 83 metaphases each) were analyzed, for the five *BRCA2*$^{+/-}$ cell lines 394 metaphases (73 -81 metaphases each) were analyzed and for the single *BRCA2*$^{-/-}$ cell line 69 metaphases were analyzed without MMC treatment (Table 2).

No significant difference in chromatid breaks was found between the wild-type genotype (*BRCA2*$^{+/+}$) and the heterozygous genotype (*BRCA2*$^{+/-}$), p = 0.076 (Figure 10A). The single homozygous (*BRCA2*$^{-/-}$) cell line, however, showed significantly higher rates of chromatid breaks p = 0.00028 (Figure 10A). No radial formation was detected among the *BRCA*$^{+/+}$ or *BRCA*$^{+/-}$ cell lines, but a low number of radial chromosomes, 0.06 aberrations/cell, was detected in the BRCA2$^{-/-}$ cell lines (Figure 10B). Although not significant, CEF seem to show a close to significant stepwise increase in aberrations between genotypes. p=0.093 when BRCA2$^{+/+}$ is compared to BRCA2$^{+/-}$, p=0.16 between BRCA2$^{+/-}$ and BRCA2$^{-/-}$ and p=0.054 between BRCA2$^{+/+}$ and BRCA2$^{-/-}$ (Figure 10C). Somewhat similar results were seen in analysis of DNA-fragments, although not statistically significant (p=0.38) (Figure 10D). DNA-fragments were not detected in wild-type cell lines but showed stepwise increase in regard to *BRCA2* status.

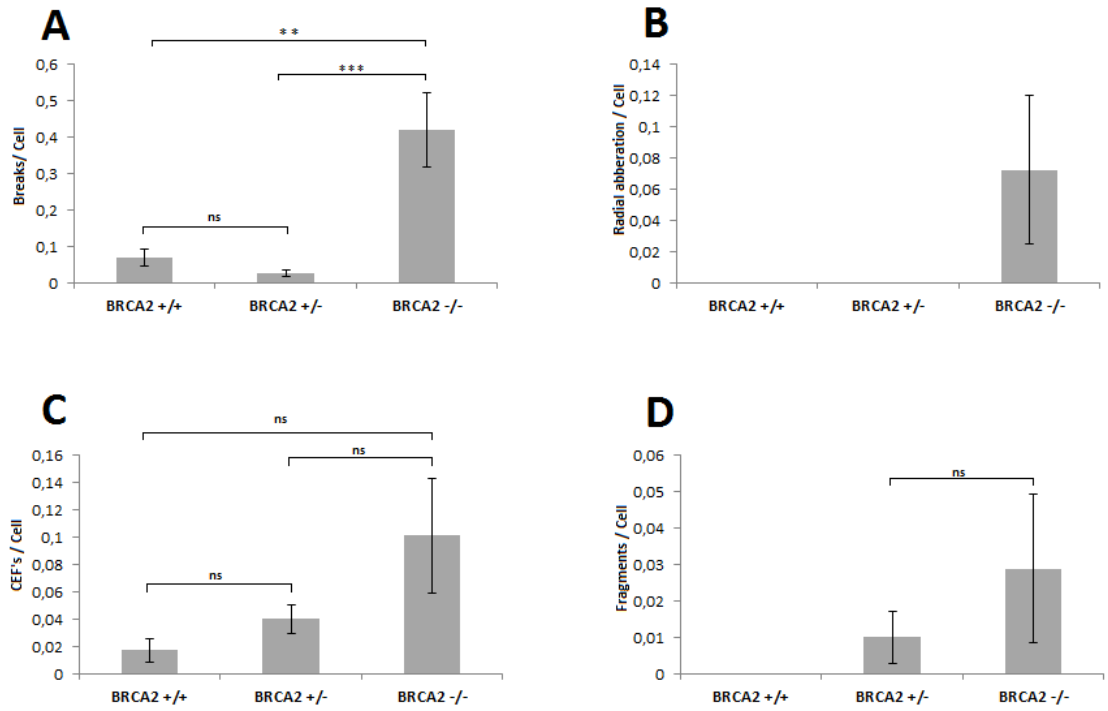

Figure 10. Chromosomal abnormalities in cell lines of different *BRCA2* genotypes when not treated with Mitomycin C. Columns are means of pooled data from each genotype with SEM bars. Chromosomal abnormalities analyzed were **A)** chromatid breaks, **B)** radial chromosome formation, **C)** chromatid end-to-end fusions (CEFs) and **D)** chromosome fragments. Total score of metaphases were n=228 for *BRCA2*$^{+/+}$, n=394 for *BRCA2*$^{+/-}$ and n= 69 for *BRCA2*$^{-/-}$. Statistically significant difference in chromosome breaks was found between the *BRCA2* homozygous cell line and the cell lines of other *BRCA2* genotypes. ns P>0.05 , * P≤ 0.05, ** P ≤ 0.01, *** P≤ 0.001, **** P ≤ 0.0001

4.1.2 Chromosomal abnormalities in different *BRCA2* genotypes after MMC treatment

For the three *BRCA2*$^{+/+}$ cell lines 222 metaphases (65 to 78 metaphases each cell line) were analyzed and 275 metaphases for the five *BRCA2*$^{+/-}$ cell lines, thereof 66-76 metaphases for three cell lines and 21-32 metaphases for two. For the single *BRCA2*$^{-/-}$ cell line 60 metaphases were analyzed (Table 2).

As seen in the untreated samples, no significant increase was found between *BRCA2*$^{+/+}$ and *BRCA2*$^{+/-}$ in any of the abnormalities analyzed; breaks (P=0.95; Figure 11A), radial formation (P=0.59; Figure 11B), CEFs (P=0.23; Figure 11C) and fragments that unexpectedly showed a slightly significant reduction (P=0,045; Figure 11D). The single *BRCA2*$^{-/-}$ cell line showed a highly significant increase in number of breaks and CEFs compared to the heterozygous (*BRCA2*$^{+/-}$) cell lines (p< 0.0001). In radial formation and DNA fragments (Figures 11A and 11C), no significant difference was found between different *BRCA2* genotypes, except fragments ranked a bit lower in *BRCA2*$^{+/-}$ (Figure 11D).

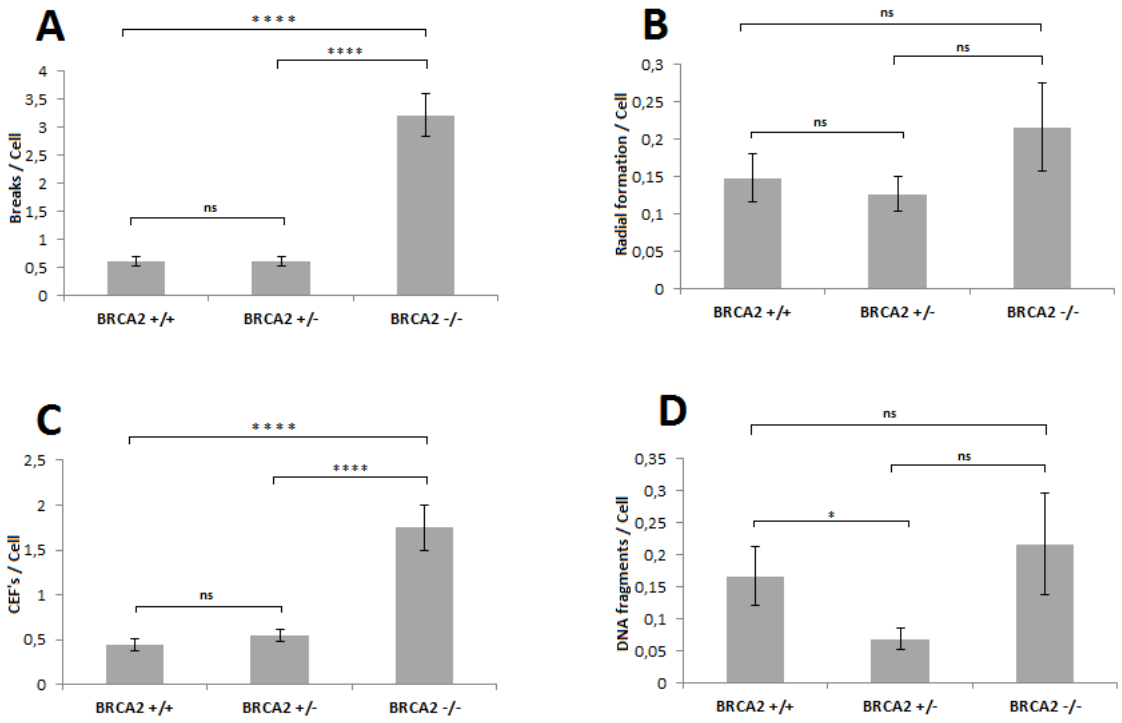

Figure 11. Chromosomal instability in cell lines of different *BRCA2* genotypes when treated with 50 ng/ml Mitomycin C. Columns are means of pooled data from each genotype with SEM bars. Chromosomal abnormalities analyzed were **A**) chromatid breaks, **B**) radial chromosome formation, **C)** chromosome end-to-end fusions (CEF) and **D)** chromosome fragments. Total score of metaphases were n = 222 for *BRCA2*$^{+/+}$, n = 275 for *BRCA2*$^{+/-}$ and n = 60 for *BRCA2*$^{-/-}$. Statistical significant increase was found in chromosome breaks and end-to-end fusions between the homozygous *BRCA2* cell line and the other genotypes. ns P>0.05 , * P≤ 0.05, ** P ≤ 0.01, *** P≤ 0.001, **** P ≤ 0.0001

4.1.3 Chromosomal abnormalities; cell line heterogeneity

Figure 12 shows chromosomal changes in all analyzed cell lines when treated with MMC (untreated not shown). It gives a view of the variation seen between individual cell lines within and of different *BRCA2* and *BRCA1* genotypes. Columns show means and SEM bars of each cell line scored. The three *BRCA2* genotype *BRCA2*$^{+/+}$, *BRCA2*$^{+/-}$ and *BRCA2*$^{-/-}$ cell lines and the two *BRCA*1$^{+/-}$ cell lines, seem to show some heterogeneity in chromosome abnormalities after treatment with 50 ng/mL MMC. Two out of the five *BRCA2* heterozygote cell lines seem to show higher number of abnormalities than other *BRCA2* heterozygotes with increased break and radial formation (Figures 12A and 12B) and three out of five show increased CEF formation compared to the wild type phenotype (Figure 12C). The single *BRCA2* $^{-/-}$ cell line shows the highest instability as was expected in formation of breaks and CEFs (Figures 12A and 12C). The two *BRCA1* heterozygote cell lines seem to show the same heterogeneity as *BRCA2*.

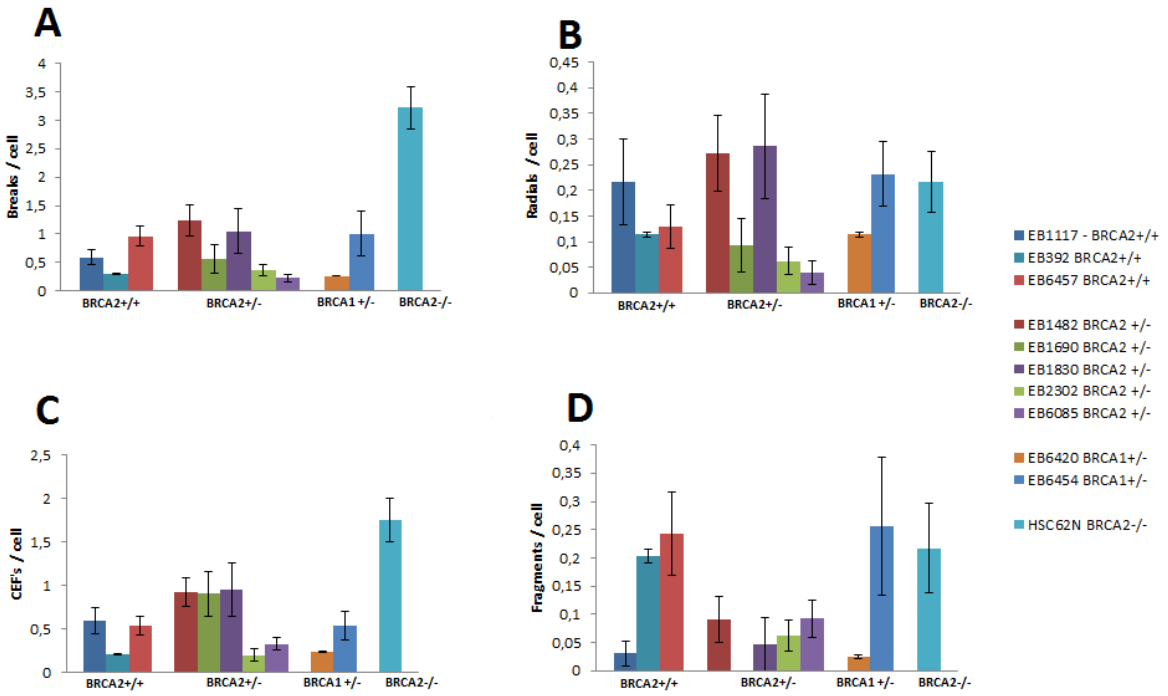

Figure 12. Chromosomal abnormalities in all cell lines analyzed after treatment with 50 ng/ml MMC (untreated not shown). Each column represent means for each individual cell line analyzed for **A)** chromatid breaks, **B)** radial chromosome formation, **C**) chromatid end-to-end fusions (CEF) and **D)** DNA fragments. Bars of each column indicate SEM.

4.2 Telomere dysfunction in lymphocyte cell lines

Metaphase spreads of five lymphocyte cell lines were analyzed for telomere dysfunction with and without 50 ng/ml MMC treatment. One *BRCA2* wild-type cell line (EB392), two *BRCA2* heterozygous cell lines (EB2302 and EB6085) and one *BRCA2* homozygous cell line the HSC62. To address telomere dysfunction four parameters were analyzed on metaphase spreads using telomere PNA probes. Parameters were a) single telomere signals, b) multiple telomere signals (MTS), c) telomere free chromosome ends and d) interstitial telomere sequences (ITS) (Figure 13). Three additional untreated *BRCA2*$^{+/-}$ cell lines were analyzed for ITS only and only when untreated (EB1690, EB1482 and EB1830; Table 3).

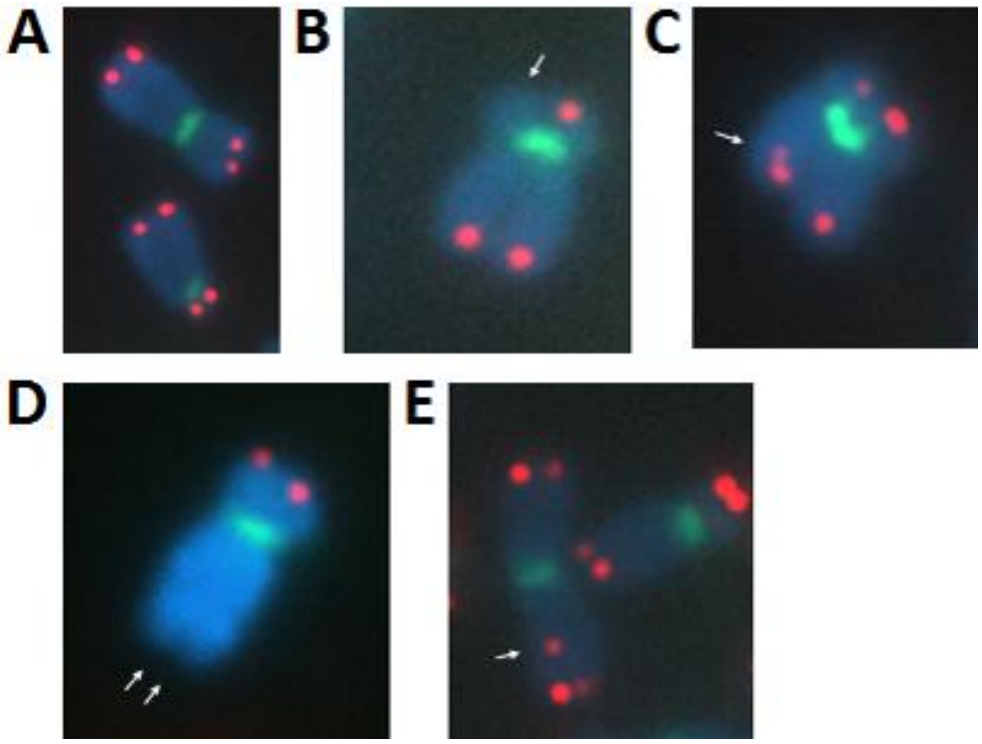

Figure 13. Telomere dysfunctions analyzed with FISH. Telomere specific red PNA-probe binds to telomeres on all chromosomes and the centromere specific green probe binds to all centromeres. Samples were mounted with DAPI-blue staining binding to all DNA. **A**. Normal chromosomes in cell line EB6085 after MMC treatment. **B** Single telomere signal in cell line EB6085 without MMC treatment. **C**. Multi telomere signal in cell line EB6085 after MMC treatment. **D**. Telomere-free chromosome end in cell line HSC62N after treatment. **E**. ITS in cell line EB6085 without MMC treatment.

Table 3. Lymphocyte cell lines harvested and analyzed for telomere dysfunction. Values are dysfunctions per metaphase. Cell lines EB1690, EB1830 and EB1482 were only analyzed for ITS and only when untreated

			Untreated - avarage per metaphase					MMC treated - avarage per metaphase			
Cell line	Genotype	Metaphases analyzed Untreated	Single telomere signal	Telomere free end	Multi telomere signal	Interstitial telomere sequence	Metaphases analyzed MMC treated	Single telomere signal	Telomere free end	Multi telomere signal	Interstitial telomere sequence
EB392	BRCA2+/+	64	5,906	1,281	3,406	0,234	57	5,088	1,298	3,404	0,404
EB2302	BRCA2+/-	62	17,548	4,435	3,823	0,306	54	22,796	6,370	5,463	2,389
EB6085	BRCA2+/-	60	12,683	2,450	4,400	1,683	62	10,355	1,484	3,532	1,258
HSC62N	BRCA2-/-	43	13,581	2,558	3,442	2,140	30	22,033	5,833	3,967	2,733
EB1690	BRCA2+/-	60	N/A	N/A	N/A	0,800	N/A	N/A	N/A	N/A	N/A
EB1830	BRCA2+/-	61	N/A	N/A	N/A	1,197	N/A	N/A	N/A	N/A	N/A
EB1482	BRCA2+/-	61	N/A	N/A	N/A	0,984	N/A	N/A	N/A	N/A	N/A

The *BRCA2* wild-type cell line EB392 showed no significant response to 50 ng/ml MMC treatment in any of the telomere dysfunction markers (Figures 14 A-D). The two *BRCA2* heterozygous cell lines however responded differently to MMC treatment. In EB2302 a significant increase was seen in all parameters including single telomere signals, MTS, telomere free chromosome ends and a dramatic increase in ITS. The EB6085 cell line showed, however, an unexpected significant decrease in all markers analyzed for unknown reasons (Figures 14 A-D). Interestingly, the initial two *BRCA2* heterozygous cell lines analyzed, EB2302 and EB6085, showed an increase in ITS compared to their wild-type counterpart when untreated and seemed to rank in between the wild-type and homozygous genotype cell lines (Figure 14D). As this was of special interest, three additional untreated heterozygous cell lines were added and analyzed for ITS only and that confirmed this observation even further (Figure 14D). The homozygous *BRCA2* $^{-/-}$ cell line, HSC62N, showed significant increase in single telomere loss, and telomere free ends as a result of MMC treatment (Figure 14 A-B) but MTS and ITS did not, although ITS was close to significance with P-value of 0,051. (Figure 14C and 14D)

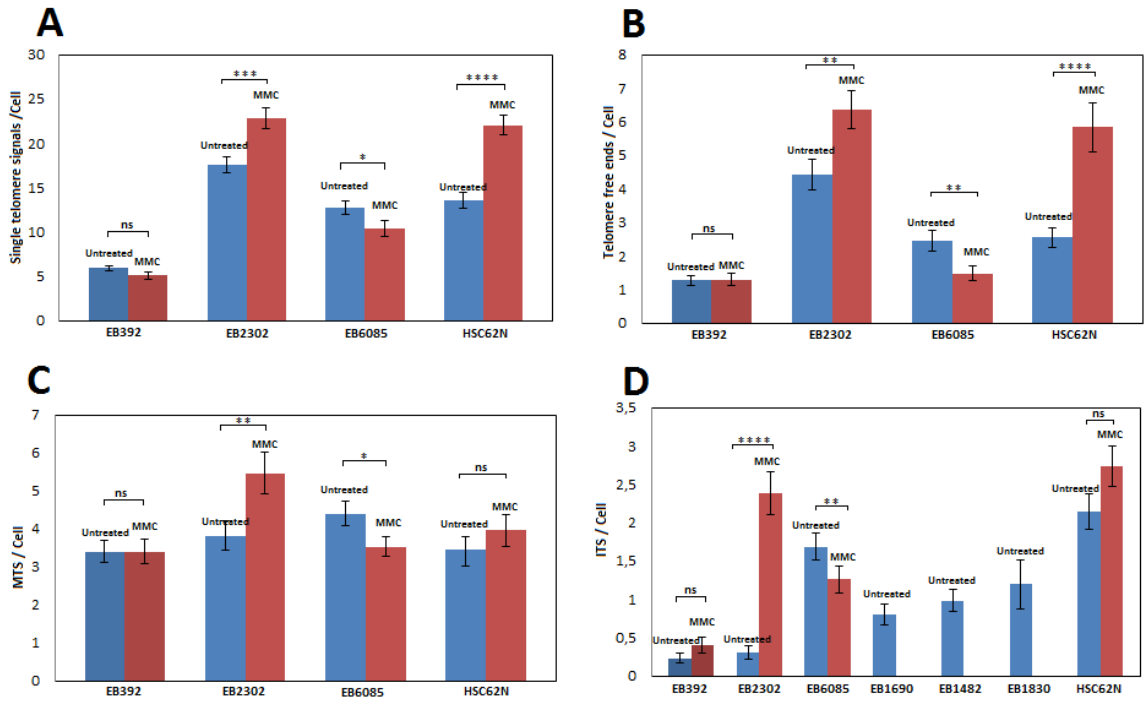

Figure 14. Telomere dysfunction analysis in lymphocyte cell lines with and without 50 ng/ml MMC treatment including **A**. single telomere signal, **B.** telomere free chromosome ends, **C**. multiple telomere signals, and **D.** interstitial telomere sequences. Blue columns refer to untreated cells and red columns refer to treated cells. Columns show means with SEM bars and significance marked as stars were ns P>0.05 , * P≤ 0.05, ** P ≤ 0.01, *** P≤ 0.001, **** P ≤ 0.0001.

4.2.1 Telomere dysfunction in different BRCA2 genotypes without MMC treatment

Telomere dysfunction was analyzed in lymphocyte cell lines of different *BRCA2* genotype without MMC treatment. One *BRCA2* wild-type cell line (EB392), two *BRCA2*$^{+/-}$ cell lines (EB2302 and EB6085) and one *BRCA2*$^{-/-}$ FANCD1 cell line (HSC62N) were analyzed. For ITS analyses, three additional *BRCA2*$^{+/-}$ cell lines were added, the EB1690, EB1482 and EB1830 cell lines (Table 3).

Single telomere signals, were signal is lost from one sister chromatid, were significantly higher in *BRCA2*$^{+/-}$ and *BRCA2*$^{-/-}$ cell lines compared to the *BRCA2* wild-type cell line. No significant difference was, however, found between *BRCA2* heterozygous and homozygous cell lines (Figure 15A).

Telomere free chromosome ends, were telomere signals are lost on both chromatids, were also significantly increased in both *BRCA2*$^{+/-}$ and *BRCA2*$^{-/-}$ cell lines compared to the wild-type cell line. Unexpectedly, a significantly fewer telomere free chromosome ends were seen in *BRCA2-/-* than in *BRCA2*$^{+/-}$ cell lines (Figure 15B). MTS were two telomere signals or telomere duplet is found on a single chromatid, were significantly higher in *BRCA2*$^{+/-}$ cells compared to either *BRCA2* wild-type or *BRCA2*$^{-/-}$ cell lines (Figure 15C). ITS, were telomere signal is found within a chromosome, were statistically significant stepwise increase was found between cell lines regarding to *BRCA2* genotype where the wild-type cell line showed the lowest and the *BRCA2*$^{-/-}$ cell line showed the highest number of ITS in cell lines (Figure 15D).

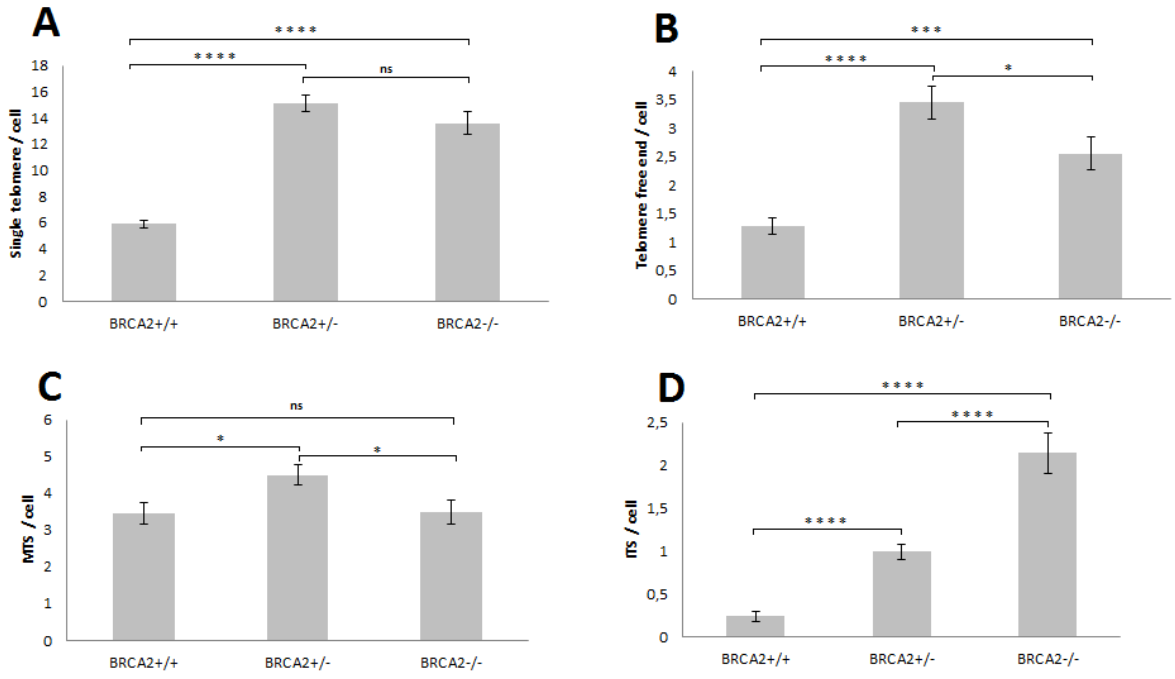

Figure 15. Telomere dysfunction in lymphocyte cell lines with different *BRCA2* genotype when untreated, Analyzed for **A)** single telomere loss, **B)** telomere free chromosome ends, **C)** multiple telomere signals and **D)** interstitial telomere sequences (ITS). Columns show average number of dysfunctions per metaphase and SEM bars after pooling the results from each genotype. Statistical significance is marked as stars where ns P>0.05 , * P≤ 0.05, ** P ≤ 0.01, *** P≤ 0.001, **** P ≤ 0.0001

4.2.2 Telomere dysfunction in different *BRCA2* genotypes after MMC treatment

Telomere abnormalities were analyzed in lymphocyte cell lines with different *BRCA2* genotype after 50 ng/ml MMC treatment followed by 72 hours cultivation. One *BRCA2* wild-type cell line (EB392), two *BRCA2*$^{+/-}$ cell lines (EB2302 and EB6085) and one *BRCA2*$^{-/-}$ FANCD1 cell line (HSC62N) were analyzed (Table 3). Significant stepwise increase was seen in single telomere loss between the *BRCA2* genotypes (Figure 16A). The same significant tendency was seen for telomere free chromosome ends (Figure 16B). MTS were slightly significantly increased in *BRCA2*$^{+/-}$ heterozygous cell lines compared to wild-type but no significant difference was seen between the homozygous genotype compared to either heterozygous or wild-type genotypes (Figure 16C). ITS had the same significant stepwise increase as single telomere loss or telomere free chromosome ends (Figure 16D).

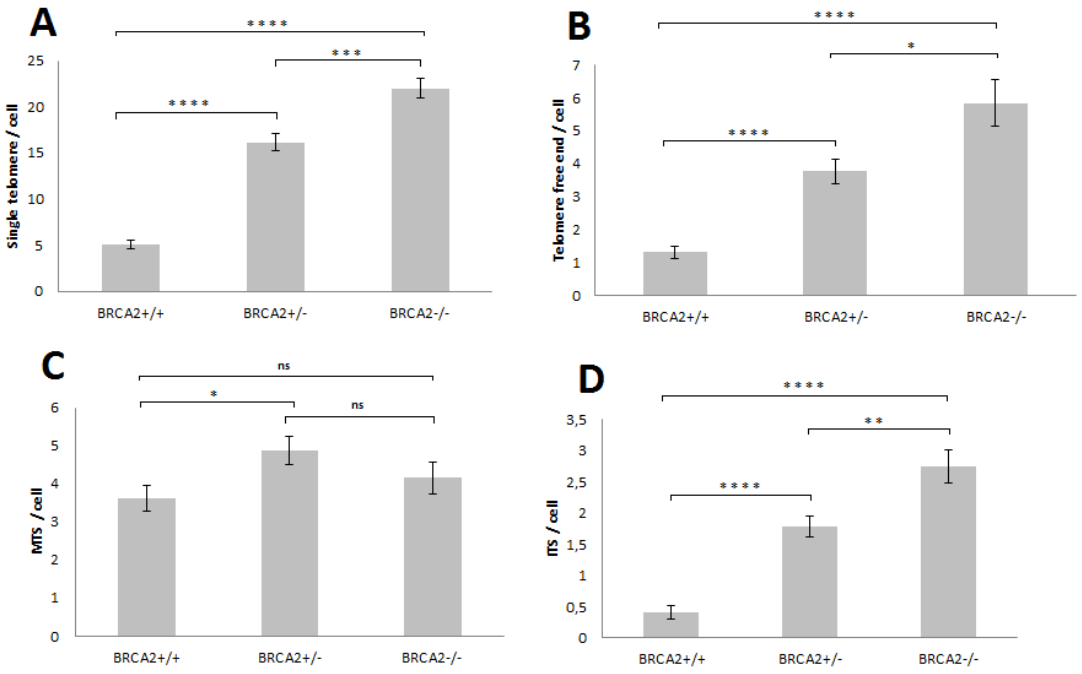

Figure 16. Telomere dysfunction in lymphocyte cell lines with different *BRCA2* genotype after treatment with 50ng/mL Mitomycin C. Average number of **A)** single telomere signals on chromosome ends, **B)** telomere free chromosome ends, **C)** multi telomere signals and **D)** interstitial telomere sequences (ITS) was analyzed for each genotype. Columns show average number of dysfunctions per metaphase and SEM bars after pooling the results from each genotype. Statistical significance is marked as stars where ns P>0.05, * P≤ 0.05, ** P ≤ 0.01, *** P≤ 0.001, **** P ≤ 0.0001.

5 Discussion

5.1 Summary

In this study *BRCA2* haploinsufficiency was addressed with respect to DNA repair and telomere maintenance. Chromosomal instability and telomere dysfunction was analyzed in EBV-transformed lymphocyte cell lines with different *BRCA2* genotype; *BRCA2* wild-type (+/+), *BRCA2* heterozygous (+/-), *BRCA2* homozygous (-/-). Cells were either untreated or challenged with a low dosage of the cross-linking agent MMC followed by 72 hour post treatment cultivation to give cell lines time to respond to the induced DNA damage. By giving the cells time for recovery the focus was set on the efficiency of response pathways dealing with DNA cross-linking repair and telomere maintenance. All cell lines showed an increase in chromosomal instabilities after MMC treatment, regardless of their *BRCA2* status (Figures 6-9). By comparing *BRCA2* genotypes a close to significant difference was seen in CEFs (Figure 10) in the untreated samples that might sever as an indication of possible haploinsufficiency. The homozygous *BRCA2*$^{-/-}$ cell line, however, showed significantly increased chromosomal abnormalities compared to wild-type and heterozygous *BRCA2* cell lines, as expected (Figures 10 and 11).

Telomere dysfunction was detected only in *BRCA2*-heterozygous and *BRCA2*-homozygous in response to MMC treatment, i.e. the wild-type cell lines remained unaffected by MMC (Figure 14). Different from the chromosomal abnormality analyzes, a significant difference in telomere dysfunction was seen between the *BRCA2* wild-type and heterozygous cell lines as well as between the heterozygous and homozygous cell lines i.e. a stepwise increase in telomere dysfunction in regard to *BRCA2* genotype. These findings were seen in both untreated and even more clearly in MMC-treated cell lines. These results not only indicate a potential role for *BRCA2* in telomere maintenance, but also suggest *BRCA2* haploinsufficiency in regard to telomere maintenance, even in untreated cells.

5.2 BRCA2 in relation to chromosomal abnormalities

In this study we show that chromosomal abnormalities were, as expected, higher in BRCA2 deficient cells (FA-D1 cells). An increase in chromosomal abnormalities was seen between *BRCA2* wild-type and *BRCA2* heterozygous cells without MMC treatment that was close to being statistically significant for CEFs and chromosome fragments (Figures 10 C and D). Although it might indicate a mild BRCA2 haploinsufficiency in regard to chromosomal maintenance, the difference was not statistically significant and also it should be kept in mind that numbers of CEFs and chromosome fragments per cell were relatively low, leaving coincidental factors playing a bigger role. Furthermore, this tendency was not seen when cells were treated with MMC and numbers of events per cell were higher. Although the FA-D1 cell line showed the highest rate of abnormalities in all parameters compared to *BRCA2* wild-type and heterozygous cell lines, it was not always statistically significant as

would be expected (Figures 10 and 11). That might be due to the fact that the FA-D1 cell line is still producing a mutated but close to a full length BRCA2 protein (Howlet *et al*., 2002). The mutated protein might still have some of its properties left based on that the individual which the cell line was derived from is known to have mild FA symptom phenotype (Howlet *et al*., 2002). The cell line has also been reported to present a lower rate of radial chromosome formation compared to other FA-D1 lymphocytes where HSC62 showed radials in 38% of cells compared to >90% in others (Howlet *et al*., 2001). That is also in correlation with a BSc-study performed at our lab where two additional FA-D1 cell lines, SPAN and NORD (also kindly provided by Helmut Hanenberg) were analyzed for telomere dysfunctions and showed more dramatic dysfunctions than the HSC62N cell line (Þorvaldsdottir, 2013). The low MMC dosage of 50 ng/ml in our study, which is consistent to a standardized FA diagnosis test, might therefore not result in a full MMC response as would be expected in a typical FA cell line.

Numerous observations have been reported linking BRCA2 to the DNA replication machinery. Pathways like HR repair of DSB, stabilization of stalled replication forks by preventing degradation of newly synthesized DNA by exonuclease MRE11 and mitotic entry control after DNA repair have all been reported to involve a role for BRCA2 (Thorlacius *et al*., 1996; Arnold *et al*., 2006; Ayoub, *et al*., 2009; Schlacher*, et al*., 2011.). Our results suggest, in lymphocytes at least, that one functional allele of *BRCA2* seems sufficient for normal efficiency of response mechanisms to maintain chromosomal stability when treated with a low dosage of a DNA crosslinking agent such as MMC. However, as the HSC62N could handle the standardized MMC dosage of the FA test as well as it did, it would be interesting to compare the response of all genotypes with a higher MMC dosage.

5.3 BRCA2 in relation to dysfunctional telomeres

In this study we found that BRCA2 deficiency is significantly associated with telomere dysfunction. The role of BRCA2 in regard to telomere length, maintenance and stabilization is not well defined and studies on telomere length in relation to breast cancer risk have been showing conflicting results. On one hand, studies show that increased telomere length is associated with increased breast cancer risk and a poor disease outcome (Gramatges *et al*., 2010; Svenson *et al*., 2008). On the other hand, results have also show no correlation is found at all (De Vivo, *et al*., 2009). Studies on *BRCA2* mutation carriers show that carriers do have longer telomeres than their non-carrier relatives, but that the telomere length does not correlate with their cancer predisposition (Pooley *et al*., 2014) or even that there is no correlation at all between *BRCA2* status and telomere length (Killick *et al*., 2014).

In the resent years, evidence linking BRCA2 function to telomere stabilization have been submerging. Our findings show that CEFs are more frequent in breast cancer from $BRCA2^{999del5}$ mutation carriers compared to non-carriers despite a normal telomere length. Moreover, epithelial cell lines carrying the $BRCA2^{999del5}$ mutation, CEFs were not only more common compared to control cell lines, but also showed occasional telomere signal at the fusion point, suggesting a telomere capping

defect related to *BRCA2* genotype (Bodvarsdottir et al., 2012; [Appendix paper 3]). Furthermore, the *BRCA2*^{999del5} heterozygous cell lines also showed more frequent T-SCE, a feature exhibited by cells using ALT which is based on recombination-mediated telomere elongation (Bodvarsdottir, et al., 2012; [Appendix paper 3]). Combined with identification of TIFs where γ-H2AX is co-localized with telomeres in those cell lines, speculations of a potential role for BRCA2 in telomere maintenance was supported even further. The absence of BRCA2 has also been reported to increase telomere dysfunction such as shortening of telomeres, CEFs and increased T-SCE in MEFs (Min, *et al*. 2012). It has been suggested that the increased number of TTAGGG repeats of telomeres impose DNA replication difficulty when the replication fork is challenged by replication of the DNA repeats and formation of the telomere G-quadruplex (G4) structures that mechanically hinder the replication process (Min, *et al*., 2012). Cellular functions involved in the unwinding of G4 structures, such as DNA repair mechanisms, might therefore be essential for normal telomere replication and maintenance. In fact, BRCA2 seems to bind to telomeres during the S and G2 phases of the cell cycle, functioning as a RAD51 loader onto telomeres during cell proliferation in MEFs (Badie *et al*., 2010). Depleting BRCA2 in MEFs was shown to result in increased TIF formation and telomere dysfunction, further establishing BRCA2 connection to telomere stabilization (Badie *et al*., 2010). This links BRCA2 strongly to telomere replication maintenance in addition to its more defined role in HR.

In this thesis, human lymphocyte cell lines showed a clear stepwise increase in distinct telomere dysfunction parameters with regard to *BRCA2* genotype when treated with MMC (Figure 16). Single telomere signals, telomere free chromosome ends and ITSs were significantly different between different *BRCA2* genotypes whereas MTS was the only parameter not significantly affected. When untreated, the *BRCA2* heterozygous and homozygous lymphocytes showed a similar rate of single telomere signals and telomere free ends (Figure 15) and differed statistically significantly from *BRCA2* wild-type genotype. This suggests that BRCA2 haploinsufficiency in its potential role in telomere maintenance might be present during cell proliferation. The fact that the *BRCA2* homozygous cell line did not show higher rate of telomere dysfunction than the heterozygous cell lines when untreated might be due to HSC62N's mild FA-D1 phenotype, as discussed before, and that its telomere maintenance deficiency is somewhat similar to *BRCA2* heterozygous cell lines when untreated. ITS however, showed a stepwise increase in regard to *BRCA2* genotypes when untreated similar to what was seen after MMC treatment.

Taken together, our results suggest that not only does BRCA2 play a role in telomere maintenance but also shows a haploinsufficient effect from carrying the *BRCA2*^{999del5} mutation, even in untreated cells (Figures 15 and 16). Furthermore, as our results from the first part of this study have shown that no significant BRCA2 haploinsufficiency was found to be associated with chromosomal stability. The BRCA2 function in telomere replication and maintenance might be suggested to be of a more delicate nature than in HR. This can lead to an interesting speculation about possible initial steps of tumor formation in BRCA2 mutation carriers through the telomere replication machinery, rather than HR repair of DSB alone as previously thought.

5.3.1 Interstitial Telomere Sequences

Increased ITSs in relation to *BRCA2* genotype has only be reported by our study and is therefore of special interest (Bodvarsdottir *et al*., 2012; [Appendix 3]). Several different molecular events are believed to lead to ITS formation. First, ITSs have been reported in several eukaryotes and assumed to be the result of CEF events during evolution or by the insertion of telomeric DNA sequences into unstable DSB sites by repair mechanisms (Azzalin *et al*., 2001). Second, BFB cycles where dicentric chromosomes are formed by telomere-to-telomere fusions, break up as chromosomes are pulled to the opposite poles during cell division, leaving a terminal deletion on one chromosome and inverted duplication containing a telomere sequence on the other (Bolzán, 2012). Third, telomere capture where telomeric DNA fragments resulting from telomere dysfunction, are inserted by NHEJ at unstable DSB sites (Bolzán, 2012). Fourth, chromosome healing were telomerase catalyzes telomeric sequences directly at or near DSB sites have been described in several species, including mammals (Melek *et al*. 1996; Bolzán, 2012). Chromosome healing has however been reported to be rare and supposedly an unlikely event (Bolzán, 2006). The presence of ITS have been linked to genome instability in humans, but the mechanism by which it affects genome instability is poorly understood (Bolsán, 2006). The BRCA2 related ITS formation seen in our study might be the result of one or more of the known pathways described above or by ways yet to be discovered. As our results show that deficiency in telomere maintenance is present and that telomeres are frequently lost on chromosome ends (Figures 15A-B and 16 A-B), it is likely that ECTR scattered around might be captured by NHEJ mechanisms at fragile DSB sites. Formation of CEFs are another possibility were unstable telomeres on chromatid ends are incorporated at DSB sites or by telomere-to-telomere fusion and formation of dicentric chromosomes leading to BFB cycles. However, in the first part of this study no significant increase in CEFs was seen in *BRCA2* heterozygous genotype cell lines compared to *BRCA2* wild-type cell lines (Figures 10C and 11C) as well as dicentric chromosomes were not frequent. This leaves the incorporation of ECTR at DSB sites the primary suspect.

The presence of ITSs in relation to *BRCA2* genotypes in our lymphocyte cell lines are interesting and suggest a *BRCA2* haploinsufficient instability in telomere maintenance even in unchallenged cell proliferation. However, the presence of ITSs in *BRCA2* mutation carriers and their potential effect on genome stability remains an unaddressed but interesting question.

5.4 Cell line heterogeneity

The lymphocyte cell lines showed some heterogeneity in both chromosomal instability and telomere dysfunction (Figures 12 and 14). By pooling results from each *BRCA2* genotype group, a median effect in each *BRCA2* genotype was found. In figures 12 and 14 it is visible that some cell lines are very sensitive to MMC treatment while others show somewhat similar results as the wild-type controls. This suggests that difference in individual genetic background plays a role in how the *BRCA2* mutation affects genetic stability through the pathways of telomere and chromosomal maintenance.

6 Conclusions

In this thesis we show that carrying the *BRCA2 999del5* mutation in one allele of the *BRCA2* gene seems to have little or no effect on chromosomal stability when compared to their wild-type counterpart in the lymphocyte cell line model used, even when cell proliferation is challenged with a low dosage of DNA crosslinking agent. However, a significant difference was seen in telomere dysfunction in the same heterozygous cell lines when compared to the wild-type genotype, even without DNA damaging treatment. This not only indicates a role for BRCA2 in telomere maintenance in addition to its more known role in HR repair, but also suggests that this potential role might be of a more delicate nature as it seems to be showing haploinsufficient effect from the *BRCA2* gene. The telomere dysfunction found seems however not to result in a dramatic chromosomal instability although CEF's and fragments in the untreated chromosome assays showed hints into that direction (Figure 10).

These results and our previous findings that loss of the *BRCA2* wild-type allele is not always present in tumors derived from *BRCA2* mutation carriers (Bjarnason, 2009; Skoulidis *et al*., 2010; [Appendix paper 1]; Stefansson, *et al*., 2011; [Appendix paper 2]) support the possibility that insufficiency in BRCA2's contribution to telomere maintenance could be a factor in tumor initiation and development. Furthermore, cell line heterogeneity in response to MMC (Figures 12 and 14) suggests that different genetic background might play a role in BRCA2 efficiency and whether haploinsufficient effects are present or not. That is in correlation with findings showing that some individual mutation carriers seem not to be as prone to develop cancer as others and difference in susceptibility among families carrying the *BRCA2*^{999del5} mutation.

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7 Appendix / Published papers

7.1 Appendix paper 1

Skoulidis F, Cassidy LD, Pisupati V, Jonasson JG, **Bjarnason H**, Eyfjord JE, Karreth FA, Lim M, Barber LM, Clatworthy SA, Davies SE, Olive KP, Tuveson DA, Venkitaraman AR. (2010). Germline Brca2 heterozygosity promotes Kras(G12D) -driven carcinogenesis in a murine model of familial pancreatic cancer. *Cancer Cell*,18 (5), 499-509.

7.2 Appendix paper 2

Stefansson OA, Jonasson JG, Olafsdottir K, **Bjarnason H**, Th Johannsson O, Bodvarsdottir SK, Valgeirsdottir S, Eyfjord JE. (2011) Genomic and phenotypic analysis of BRCA2 mutated breast cancers reveals co-occurring changes linked to progression. *Breast Cancer Res*, 13 (5),R95.

7.3 Appendix paper 3

Bodvarsdottir SK, Steinarsdottir M, **Bjarnason H**, Eyfjord JE. (2012). Dysfunctional telomeres in human *BRCA2* mutated breast tumors and cell lines. *Mutat Res*, 729 (1-2), 90-9.