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DNA damage and genetic polymorphisms: influence on individual radiosensitivity

Danno al DNA e polimorfismi genetici:
influenza sulla radiosensibilità individuale

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INDEX

ABBREVIATIONS pg.5

SUMMARY pg.7

I. INTRODUCTION

1. DNA damage and DNA repair pathways pg.15

1.1 MisMatch Repair (MMR) pg.15

1.2 Base Excision Repair (BER) pg.17

1.2.1 XRCC1 pg.22

1.3 Nucleotide Excision Repair (NER) pg.23

1.4 Double Strand Breaks repair: Non-Homologous End Joining (NHEJ) and Homologous Recombination Repair (HRR) pg.25

2. Gene polymorphisms and individual susceptibility pg.30

3. Radiosensitivity pg.33

3.1 XRCC1 and radiation exposure pg.34

II. AIM of the RESEARCH pg.39

III. RESULTS

1. Genetic polymorphisms, repair capacity and breast cancer risk pg.43

2. RAD51 expression and IR exposure pg.53

3. XRCC1 expression and IR exposure pg.57

IV. DISCUSSION and CONCLUSION pg.63

V. BIBLIOGRAPHY pg.71

ABBREVIATIONS

3'UTR: 3'Untranslated Region
5'dRP: 5' deoxyRibose Phosphate
5'UTR: 5' Untranslated Region
8-oxoG: 8-oxoguanine
AA: Amino-acid
AP: abasic (apurinic/apyrimidinic) site
APE1: AP endonuclease 1
AT: Ataxia Telangiectasia
ATLD: Ataxia Telangiectasia like Disorder
ATM: Ataxia Telangiectasia Mutated
ATR: Ataxia Telangiectasia and Rad3 related
BC: Breast Cancer
BER: Base Excision Repair
BRCA: Breast cancer susceptibility protein
BRCT: BRCA1 Carboxy Terminal domain
BS: Bloom's Syndrome
CHO: Chinese Hamster Ovary
CI: Confidence Interval
Ct: Threshold Cycle
CS: Cockayne Syndrome
DAR: transcription Domain-Associated Repair
DNA-PK: DNA-dependent Protein Kinase
DNA Pol: DNA Polymerase
DSB: Double Strand Break
FCA: Flow Cytometric Analysis
G0: absence of radiation effect
G1-G2-G3: grades of radiation effect
GGR: Global Genome Repair
global rapid SSBR: global rapid single strand break repair
HR: Homologous Recombination
HRR: Homologous Recombination Repair
IR: Ionizing Radiation
LCL: Lymphoblastoid Cell Line
Lig: Ligase
LP-BER: Long Patch-Base Excision Repair
miR34a*: microRNA (miR) 34a*
MMR: MisMatch Repair
NBS: Ninmegen Breakage Syndrome
NER: Nucleotide Excision Repair

NHEJ: Non-Homologous End Joining
NIR: Nucleotide Incision Repair
OGG1: 8-oxoguanine-DNA-glycosylase
PARP: Poly(ADP-Ribose)Polymerase
PBL: Peripheral Blood Leucocytes
PBMC: Peripheral Blood Mononuclear Cell
PCNA: Proliferating Cell Nuclear Antigen
PCR: polymerase chain reaction
PNK: PolyNucleotide Kinase
PNKP: PolyNucleotide Kinase 3'-Phosphatase
OR: Odds Ratio
RAD51: RAD51 homolog (RecA homolog , *E.coli*)
RD: Residual Damage
RFLP-PCR: Restriction Fragment Length Polymorphism-Polymerase Chain Reaction
ROS: Reactive Oxygen Species
RPA: Replication Protein A
RQ: Relative Quantitation
RT: Radiotherapy
RTOG: Radiation Therapy Oncology Group
RT-qPCR: quantitative reverse transcriptase-PCR
qPCR: Quantitative PCR
S/G2-specific XRCC1-dependent SSBR: S/ G2-specific XRCC1-dependent SSBR
SE: Standard error
SNP: Single Nucleotide Polymorphism
SP-BER: Short Patch-Base Excision Repair
SSA: Single Strand Annealing
SSB: Single Strand Break
SSBR: Single Strand Break Repair
TCR: Transcription-Coupled Repair
TD: Tail DNA
TFIIH: Transcription Factor IIH
UV: Ultra Violet
VNTR: Variable Number of Tandem Repeats
XP: Xeroderma Pigmentosum
XRCC1: X-ray Repair Cross-Complementing group 1
XRCC3: X-ray Repair Cross Complementing group 3
WS: Werner’s Syndrome

SUMMARY

Polymorphisms represent the main source of genetic inter-individual variability. The presence of polymorphic alleles in DNA repair genes may alter repair capacity and thus modify the biological responses to exogenous and endogenous DNA insults, both at the cellular and tissue level. In addition to impaired DNA repair capacity and increased mutagenesis, polymorphisms in DNA repair genes may also result in a modified risk of developing cancer. Radio-induced DNA damage and its repair also play a critical role in the susceptibility of patients to develop side effects after radiotherapy (RT). Therefore, the development of *in vitro* cellular radiosensitivity tests and genetic markers, that can be used as biomarkers for the extent of patients' normal tissue reactions, is of great interest. Such markers could be used to adjust RT protocols for both radio-sensitive and radio-resistant patients.

The aim of this PhD project was to analyse the relationship between induced DNA damage, the DNA damage responses and the individual's genetic background. In particular the influence of variant alleles in damage signalling (*RAD51*) and repair (*XRCC1, OGG1* and *XRCC3*) genes on individual susceptibility to developing cancer and on sensitivity to IR-exposure, were assessed analysing both *in vivo/ex vivo* and *in vitro* systems. *Ex vivo* studies were focused on breast cancer patients, enrolled in Italian and French Oncology Units and in order to investigate the cellular response to IR exposure and to find a possible explanation for differences in radiosensitivity, we conducted *in vitro* assays on lymphoblastoid cell lines (LCLs) established from BC subjects, peripheral blood mononuclear cells (PBMCs) isolated from healthy donors and the hamster (CHO) cell lines AA8 and EM9, that represent a model to study the functional role of the *XRCC1* gene.
In the research part on BC patients, the Comet assay revealed that the cases exhibited a higher level of basal and X-ray (2Gy) induced-DNA damage than healthy controls. Moreover, in patients showing no adverse reactions (G0) the DNA damage significantly decreased from 30 to 60 min of repair times, unlike BC subjects showing acute skin reactions (G1-G3).
With respect to the polymorphisms in the *XRCC1* gene, *XRCC1-399* (rs25487) was significantly associated with an increased risk of developing sporadic breast cancer. The 399-Gln may act as a dominant allele and when combined with the wild type allele at codon 194 and the variant allele at the position -77, was associated with a significantly higher BC risk. On the contrary, *XRCC1-77*, *XRCC1-194*, *OGG1-326*, *XRCC3-241*, *RAD51-01*,

RAD51-52 as individual SNPs did not show any association with BC risk. However, carrying combination of SNPs in several genes, involved in different repair mechanisms, increased the risk of developing breast cancer. We found a significantly higher BC risk for subjects with ≥3 variant alleles compared to those with <3 variants, suggesting a joint or additive effects of genetic variants in multiple repair pathways.
Using LCLs we demonstrated that *RAD51* mRNA and the microRNA (miR) 34a* were expressed constitutively and that after IR exposure (5Gy of γ-rays) they appeared induced at 2h and 4h respectively, but this induction was independent of the *RAD51-52* (rs11855560) genotype. Furthermore, by 4h to 8h post-irradiation a decrease in *RAD51* mRNA expression was noted in all the LCLs.
Differences in the constitutive levels of RAD51 protein levels were found in the four LCLs examined that also appeared to be independent of the *RAD51-52* genotype, however p53 protein levels were similar. Following IR treatment, as expected p53 levels increased reaching a maximum at 4h post-treatment, however no marked differences in RAD51 protein levels were observed.
Using the two hamster cell lines, AA8 and EM9, we investigated the impact of irradiation on XRCC1 levels. Immediately after exposure to 1.25, 2.5 and 5Gy no significant change in *XRCC1* mRNA levels was found indicating that these doses of X-rays did not cause a direct damage to RNA molecules. In contrast, western blotting analysis conducted on protein extracts from AA8 cells revealed that XRCC1 protein levels seemed to be unchanged immediately after irradiation with 1.25 and 2.5Gy but reduced immediately after 5Gy treatment. In all extracts from EM9 cells the XRCC1 protein was completely absent confirming its status as a null mutant line. In EM9 cells, which are capable of expressing *XRCC1* mRNA, the XRCC1 protein is absent as result of a C→T substitution at nucleotide 661 that introduces a termination codon thus producing a truncated polypeptide lacking two thirds of the normal sequence.
However, by comparing the *XRCC1* mRNA levels in AA8 and EM9 cells, the null mutant EM9 displayed significantly lower levels of *XRCC1* transcript than the wild type AA8, both before and immediately after treatments. It is likely that the lack of functional XRCC1 protein influenced *XRCC1* gene expression or that the small amount of *XRCC1* transcript was a consequence of a nonsense-mediated mRNA decay in EM9 cells.
Using synchronized cell lines we examined *XRCC1* mRNA levels in different cell cycle phases; in untreated AA8 cells, we observed significantly higher levels of *XRCC1* transcript in S phase compared to G_0 and G_1 and significantly reduced levels in G_1 phase when compared to S

and G_0 phases. The EM9 cells also showed a significant decrease of *XRCC1* mRNA levels in G_1 as regards G_0. In contrast, the EM9 cells did not show an increase of *XRCC1* mRNA during the replicative phase and instead they showed a decrease when compared to G_0 cells. The treatment of cells with IR (2Gy of X-rays) did not influence *XRCC1* mRNA levels in the different cell cycle phases either in AA8 or EM9 cells, except for a significant decrease in S phase in AA8 cells.

In quiescent PBMCs, we observed that IR treatment specifically caused a *XRCC1* induction in a time-dependent manner; 90 min after irradiation a significant increase of *XRCC1* mRNA levels was found in comparison to control level. However, already at 60 min post-treatment a significant, but less pronounced, enhancement in *XRCC1* expression was noted. With respect to the repair kinetics of radio-induced DNA damage, in G_0 PBMCs from 15 to 90 min after treatment a gradual and significant decrease of Tail DNA (TD) mean value, measured using the Comet assay, was detected. This trend indicated that radio-induced DNA damage is repaired very quickly after IR exposure.

In summary, we highlight the potential of *XRCC1* as a possible genetic marker to assess the risk of developing sporadic breast cancer and we suggest studying it in combination with other SNPs.

The *in vitro* CHO studies allow us to conclude that *XRCC1* is expressed differentially through the cell cycle and maximally in S phase during which the XRCC1 protein assists in DNA replication. Furthermore, by dose-response analysis, we show that the average X-ray dose generally used as a single fraction dose in radiotherapy does not affect XRCC1 mRNA and protein levels.

Investigating the response to IR in quiescent peripheral blood mononuclear cells, we can confirm that X-ray treatment causes an induction of *XRCC1* gene expression and that the DNA radio-induced damage is quickly repaired, mainly by global rapid SSBR pathway in which XRCC1 operates as a scaffold protein.

In LCLs, we conclude that the miR34a* binding in the 3'UTR of *RAD51* is not influenced by the *RAD51-52* SNP, and it does not modify *RAD51* mRNA levels. The IR activation of p53 is responsible for the induction of the miR34a* expression, seen 4h post-treatment, and for the decrease in the *RAD51* mRNA levels, observed starting from 4h post-irradiation.

I polimorfismi genetici rappresentano la principale fonte di variabilità inter-individuale. La loro presenza, sopratutto in geni implicati nella riparazione del danno al DNA, potrebbe alterare la capacità ripartiva e quindi modificare, sia a livello cellulare che tissutale, le risposte dei sistemi biologici agli insulti di tipo endogeno ed esogeno. A causa di una possibile compromissione della capacità ripartiva e quindi di un aumento del rischio di insorgenza di mutazioni, i polimorfismi nei geni coinvolti nella riparazione del danno al DNA potrebbero modificare il rischio individuale di sviluppare il cancro. Fatte queste premesse e considerando la riparazione del danno al DNA radio-indotto cruciale nel determinare la suscettibilità dei pazienti oncologici alle reazioni avverse al trattamento radioterapico, è molto importante riuscire ad individuare dei test *in vitro* in grado di misurare la radiosensibilità cellulare e dei marcatori genetici che possano essere utilizzati come indicatori dell'entità delle reazioni avverse nei tessuti normali. Tali marcatori consentirebbero di sviluppare dei protocolli di radioterapia quanto più mirati e specifici per i pazienti radio-sensibili e radio-resistenti.

Lo scopo di questo progetto di ricerca è stato quello di studiare l'influenza del background genetico individuale sulle risposte cellulari attivate in seguito all'induzione di danno a livello del DNA. In primo luogo ci siamo proposti di valutare l'influenza di varianti polimorfiche in geni coinvolti nella segnalazione (*RAD51*) e riparazione (*XRCC1, OGG1* e *XRCC3*) del danno al DNA, sulla suscettibilità individuale a sviluppare il cancro e sulla diversa sensibilità all'esposizione alle radiazioni ionizzanti. Per raggiungere questo obiettivo abbiamo effettuato studi in sistemi sia *in vivo/ex vivo* che *in vitro*.

Gli studi *ex vivo* sono stati condotti su pazienti affetti da tumore al seno, reclutati presso le Unità ospedaliere di Oncologia in Italia e in Francia. Inoltre per studiare le risposte cellulari attivate in seguito al trattamento con IR, e trovare quindi una possibile spiegazione alle differenze in termini di radiosensibilità, abbiamo effettuato esperimenti *in vitro* in linee linfoblastoidi (LCLs), derivanti da soggetti con tumore al seno, in cellule mononucleate di sangue periferico isolate da donatori sani e linee cellulari di ovario di hamster cinese (CHO) AA8 e EM9, che rappresentano un modello per studiare il ruolo funzionale del gene *XRCC1*.

Relativamente alla parte di ricerca incentrata sui pazienti con tumore al seno, il saggio della cometa (Comet assay) ha evidenziato che quest'ultimi presentano un danno a livello basale e indotto dal trattamento con raggi X (2Gy) più elevato rispetto ai controlli sani. Inoltre, nei pazienti che non hanno sviluppato reazioni avverse (G0) alla radioterapia il danno al DNA risulta significativamente ridotto tra 30 e 60 min dopo il trattamento, al

contrario dei soggetti con reazioni avverse acute a livello della pelle (G1-G3), che invece mostrano un danno residuo maggiore.
Per quanto riguarda l'analisi dei polimorfismi genetici di *XRCC1*, *XRCC1-399* (rs25487) è risultato essere significativamente associato con l'insorgenza di tumore al seno di tipo sporadico. L'allele variante *399-Gln* inoltre sembra comportarsi da allele dominante e quando è in combinazione con l'allele wild type nel codone 194 e la variante polimorfica in posizione -77 determina un significativo e più elevato rischio di sviluppare il tumore al seno. Al contrario di *XRCC1-399*, per i polimorfismi *XRCC1-77*, *XRCC1-194*, *OGG1-326*, *XRCC3-241*, *RAD51-01* e *RAD51-52,* non si è evidenziato nessun tipo di associazione con l'insorgenza di tumore al seno, quando sono stati considerati singolarmente.
Tuttavia la combinazione di varianti polimorfiche in diversi geni coinvolti nei processi di riparazione aumenta il rischio di sviluppare questo tipo di tumore. Infatti, abbiamo osservato che i soggetti che presentano tre o più varianti polimorfiche hanno un rischio significativamente maggiore di sviluppare il tumore al seno rispetto a quelli con meno di tre varianti alleliche, suggerendo quindi un effetto combinatorio o sommatorio delle varianti alleliche presenti in più meccanismi di riparazione.
Dallo studio effettuato sulle linee linfoblastoidi i nostri risultati dimostrano che *RAD51* e il microRNA 34a* sono espressi costitutivamente e in seguito all'esposizione con IR (5Gy raggi gamma) essi appaiono indotti rispettivamente a 2h e 4h dal trattamento, ma l'induzione risulta indipendente dal genotipo cellulare nel *locus RAD51-52*. Inoltre in tutte e quattro le linee linfoblastoidi è possibile evidenziare una riduzione del messaggero di *RAD51* 4-8h dopo l'irraggiamento.
A livello dell'espressione basale della proteina RAD51 sono state osservate differenze nelle quattro linee linfoblastoidi studiate, che tuttavia non risultano imputabili al genotipo cellulare. La proteina p53, invece, risulta espressa in modo simile nelle diverse linee. In risposta al trattamento con 5Gy di raggi gamma, come atteso p53 viene espressa ai massimi livelli a 4h post-irraggiamento; nessun cambiamento rilevante è stato osservato nell'espressione della proteina RAD51.
Nelle linee cellulari di hamster, AA8 e EM9, il trattamento con tre differenti dosi di raggi X: 1.25, 2.5 e 5Gy non determina immediatamente dei cambiamenti significativi a livello del trascritto di *XRCC1*, indicando quindi che queste dosi non causano un danno diretto alle molecole di RNA. L'analisi mediante western blotting ha invece evidenziato che nelle AA8 i livelli della proteina XRCC1 sembrano rimanere invariati subito dopo l'esposizione a 1.25 e 2.5Gy ma ridotti in seguito al trattamento con 5Gy. Contrariamente, nelle EM9 la proteina XRCC1 risulta completamente

assente sia nelle cellule non trattate che irraggiate, confermando quindi questa linea difettiva per XRCC1. Nelle EM9, sebbene il gene è espresso la proteina XRCC1 è assente a causa di una sostituzione C→T al nucleotide 661 che introduce un codone di stop. Questa mutazione non-senso determina la sintesi di una proteina tronca che manca dei due terzi della sequenza aminoacidica.

Attraverso il confronto dei livelli del trascritto di *XRCC1* nelle AA8 e EM9, le EM9 presentano dei livelli di *XRCC1* mRNA significativamente più bassi rispetto al controllo positivo, AA8, sia prima che immediatamente dopo i trattamenti con le tre dosi di raggi X. E' probabile che l'assenza della proteina XRCC1 funzionale influenzi l'espressione genica oppure che la ridotta quantità di messaggero di *XRCC1* sia una conseguenza del decadimento del mRNA mediato da codoni non-senso nelle cellule EM9.

Lo studio nelle linee CHO sincronizzate ci ha permesso di osservare che i livelli del messaggero di *XRCC1* risultano variare significativamente nelle differenti fasi del ciclo cellulare; nelle AA8 non trattate, noi abbiamo rilevato dei livelli del trascritto significativamente più alti nella fase S rispetto alle fasi G_0 e G_1 e significativamente ridotti in G_1 quando confrontati con S e G_0. Anche nelle EM9 i livelli di trascritto sono significativamente minori in G_1 rispetto G_0; ma, al contrario, le EM9 non esibiscono un incremento del messaggero di *XRCC1* durante la fase replicativa in cui sembra piuttosto esserci una riduzione quando confrontata con i livelli rilevati in G_0. Il trattamento con raggi X (2Gy) nelle differenti fasi del ciclo cellulare non determina una variazione dei livelli del messaggero di *XRCC1*, ad eccezione di una riduzione osservata nella fase S nelle cellule AA8.

Riguardo gli esperimenti condotti su colture di cellule mononucleate di sangue periferico quiescenti, abbiamo evidenziato che l'esposizione alle radiazioni ionizzanti causa un'induzione dell'espressione genica di *XRCC1* in maniera tempo-dipendente; a 90 min dal trattamento (2Gy raggi X) si può evidenziare un significativo incremento dei livelli del trascritto di *XRCC1* rispetto alla condizione di controllo. Tuttavia, già a 60 min post-irraggiamento un significativo, ma meno pronunciato, incremento di *XRCC1* mRNA può essere rilevato.

Per quanto riguarda la cinetica di riparazione del danno al DNA radio-indotto, nelle cellule mononucleate (PBMCs) quiescienti dai 15 ai 90 min dopo l'esposizione è possibile notare una graduale e significativa riduzione del valore medio di Tail DNA (TD), misurato attraverso il saggio della cometa. Questo andamento ci suggerisce che il danno al DNA indotto dalle radiazioni ionizzanti inizia ad essere riparato rapidamente dopo l'esposizione.

Alla fine di questi tre anni di ricerca, noi dunque evidenziamo *XRCC1* come un possibile marcatore genetico per valutare il rischio di sviluppare il tumore al seno di tipo sporadico e in particolare suggeriamo di analizzare le varianti alleliche di questo gene in combinazione con altri polimorfismi.
Inoltre, gli studi *in vitro* condotti sulle CHO ci permettono di concludere che *XRCC1* viene espresso differentemente durante il ciclo cellulare e specificatamente ai massimi livelli nella fase S, durante la quale la proteina XRCC1 sembra assistere la replicazione del DNA. In aggiunta, gli esperimenti di dose-risposta hanno evidenziato che la dose media di raggi X generalmente utilizzata come singola frazione in radioterapia (2Gy) non causa un danno diretto a livello delle molecole di RNA.
Lo studio delle risposte cellulari alle radiazioni ionizzanti nelle cellule mononucleate di sangue periferico quiescenti ci permette di affermare che il trattamento con i raggi X (2Gy) è responsabile di un'induzione dell'espressione genica di *XRCC1* dipendente dal tempo e che il danno al DNA radio-indotto viene riparato in tempi precoci. La riparazione riteniamo avvenga principalmente attraverso il processo *global rapid SSBR (single strand break repair)* in cui XRCC1 opera come proteina scaffold coordinando il reclutamento delle diverse componenti che vi intervengono per riparare la lesione al DNA.
Nelle linee cellulari linfoblastoidi possiamo concludere che il legame del microRNA34a* al 3'UTR del gene *RAD51* non risulta influenzato dal polimorfismo *RAD51-52*, e che il miR34a* non modula i livelli di trascritto di *RAD51*. L'attivazione di p53, conseguente al trattamento con radiazioni ionizzanti, è responsabile dell'induzione dell'espressione del miR34a*, osservata a 4h post-trattamento, e della riduzione dei livelli del messaggero di *RAD51* registrati 4-8h dopo l'irraggiamento.

I. INTRODUCTION

1. DNA damage and DNA repair pathways

During evolution, mammals have evolved distinct pathways to repair their DNA thus preserving genome integrity and avoiding generating and fixing harmful mutations that could promote the onset of several diseases.

DNA lesions could be caused by exposure to external agents, such as ionizing and UV radiation and mutagenic substances such as those found in tobacco smoke but they can also be produced endogenously, for example by reactive oxygen species (ROS) generated during physiological processes. Protein sensors, such as ATM and ATR detect the DNA lesions and trigger a series of cellular responses including, firstly, DNA repair.

In human cells five different repair mechanisms have been well described: MMR (MisMatch Repair), BER (Base Excision Repair), NER (Nucleotide Excision Repair), HRR (Homologous Recombination Repair) and NHEJ (Non-Homologous End Joining). Each of these pathways, specialized in removing or correcting different kinds of DNA lesions, are a finely regulated step-by-step process.

Figure 1 illustrates schematically the link between damaging agents and the relative DNA repair pathways that can be activated in response to different DNA lesions. In some case, it is possible that there is an overlap of the different repair processes and a cross-talk between them, to optimize and safeguard the cell's viability.

1.1 Mismatch Repair (MMR)

MMR is a postreplicative mechanism that ensures the application of Watson-Crick base pairing principle of the DNA double helix, by discriminating mismatches resulting from DNA polymerase errors and rectifying them to avoid mutations being propagated into daughter cells.

Even if DNA polymerases have a high fidelity and in particular for Polδ and Polε it is further improved to 1 in 10^7 nucleotides synthesized by their inherent proofreading ability (McCulloch and Kunkel, 2008), DNA biosynthesis represents a source of mismatches.

Failure of mismatch correction will give rise to genetic mutations: purine-pyrimidine mismatches will generate transition mutations in 50% of the newly synthesized DNA and purine-purine or pyrimidine-pyrimidine mismatches transversion mutations.

Therefore, to prevent DNA mutations cells monitor and assist DNA replication through MMR. The eukaryotic MMR, in fact, depends on factors

which are components of the replication machinery (Kleczkowska *et al.*, 2001; Johnson and O'Donnell, 2005), as outlined below.

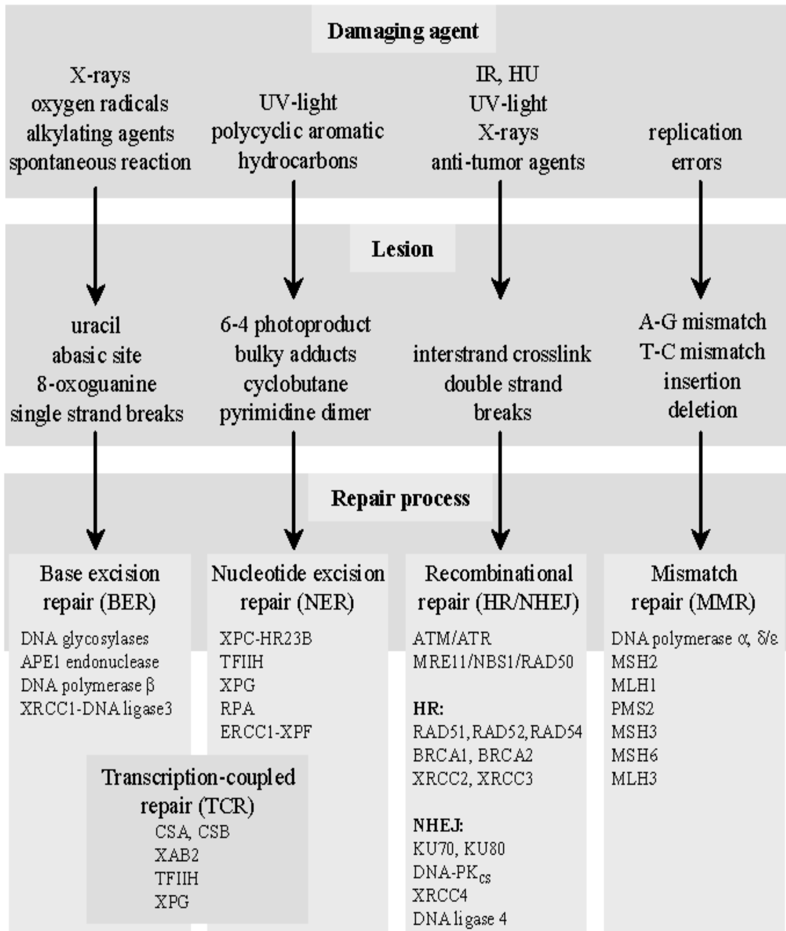

Fig.1 A simplified schema of the most important repair processes. DNA-damaging agents (top), examples of DNA lesions (middle), and the relevant repair mechanisms (bottom). The essential genes involved in each DNA repair pathway are shown below the corresponding titles. (Adapted from Hoeijmakers., 2001, Khanna and Jackson 2001, Svejstrup, 2002)

Briefly, in eukaryotes an occasional base-base mismatch is recognized by MutSα, an heterodimeric complex composed of MSH2 and MSH6, which possesses ATPase activity. The mismatch recognition depends on increased DNA pliability caused by the helix destabilizing effect of the mispair, as proposed by Schofield and Hsieh (2003). Then, MutSα recruits the MutLα heterodimer (MLH1+PMS2) and after to an ADP→ATP exchange, that induces a conformational change, this quaternary complex is able to slide away from the mismatch along the DNA duplex. Hence, MutLα actived by MutSα and some components of the replication apparatus (PCNA, RFC), introduces nicks up- and down-stream of the mismatch in the newly synthesized filament.
The nascent DNA strand is discriminated by the presence of DNA primer ends and strand-interruptions between unprocessed Okasaki fragments.
Subsequently, the nuclease EXO1 excises the tract containing the mispair thus generating a gap that is filled in by Polδ. Finally, DNA ligase I completes repair by sealing the nick. (Figure 2)
Unlike DNA polymerases errors that occur mainly in the nascent strand during replication, mismatches can also be induced in either strands by chemical (i.e. alkylating agents) or physical mutagens which modify the Watson-Crick hydrogen-bonding partner.
In this case, mismatches caused by DNA base modifications are recognized by specific and selective DNA glycosylases which catalyze the hydrolysis of the N-glycosidic bond of a damaged deoxynucleoside generating an abasic site (AP), so initiating the BER (Base Excision Repair) process.

1.2 Base Excision Repair (BER)

BER is the most versatile and the predominant DNA repair pathway for small base lesions, caused by oxidative, alkylation and deamination damage (Lindahl, 1993; Kavli *et al.,* 2007; Sedgwick *et al.*, 2007). Moreover, single strand breaks (SSBs) generated by reactive oxygen species (ROS), byproducts of metabolism and irradiation (X- and γ-rays) are repaired by single strand breaks repair (SSBR), a DNA repair pathway that utilizes the BER proteins but differs from it in the recognition initial step. BER corrects DNA base lesions via two sub-pathways: short patch (SP-BER), a mechanism whereby only 1 nucleotide is replaced and long patch (LP-BER) that leads to a repair tract of at least two nucleotides.
For both, the first step consists of the recognition of a damaged base by an appropriate DNA glycosylase which determines the specificity of this pathway. (Robertson *et al.*, 2009)

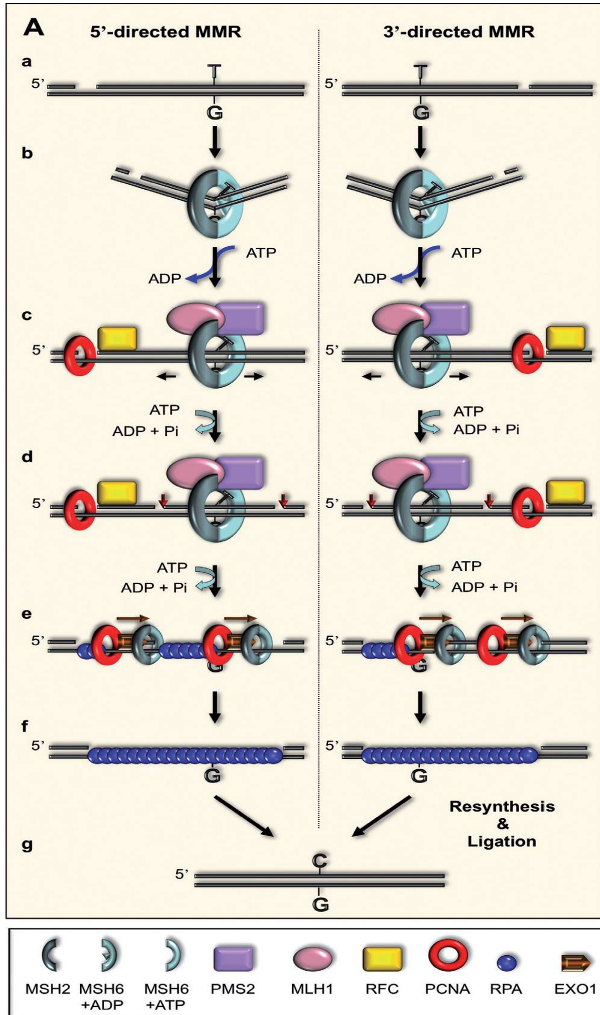

Fig.2 Simplified overview of MisMatch Repair in eukaryotics. Briefly, MSH2 and MSH6 compose MutSα heterodimer which binds the mismatch (b) and recruits the MLH1-PMS2 heterodimer (MutLα) (c). This latter when activated is able to introduce nicks in the discontinuous strand (red arrowhead) (d) and the nicked strand is degraded by EXO1 (e), generating single gaps which are protected by RPA (f). Repair is completed by polymerase and ligase. (Modified from Kunz *et al*., 2009)

Basically, the BER pathway, discovered in *E.coli* nearly 35 years ago by Lindhal (1974) can be resumed in five distinct enzymatic steps, illustrated in Figure 3.
As mentioned above, the first step consists in DNA damaged base recognition by a DNA glycosylase, which could be mono- or bi-functional.
To date, 11 different mammalian glycosylases have been described (Table 1) and four of them are specialized to remove oxidized-bases: OGG1 (8-oxoguanine-DNA glycosylase) (Aburatani H *et al.*, 1997); NTH1 (homolog of the E.coli Nth endonuclease) (Ikeda S *et al.*, 1998); NEIL1 (Nei-like1) and NEIL2 (Nei-like2) (Hazra *et al.,* 2002).
OGG1, NTH1, NEIL1 and NEIL2 are bifunctional glycosylases with an additional AP lyase activity which allows them to process the abasic site (AP) via β or βδ elimination reaction. However, for OGG1 which excises 8-oxoguanine (8-oxoG), resulting from the oxidative damage of G base, the lyase reaction is very weak (Hill *et al.*, 2001).

Table 1 DNA glycosylases and their enzymatic activities (human nomenclature)

ENZYME	TYPE*	ENZYMATIC ACTIVITIES
UNG	M	Uracil
SMUG1	M	uracil, 5-OH-meU
TDG	M	T, U and ethanoC (CpG sites)
MDB4	M	T and U opposite G (CpG sies)
MUTYH	M	A opposite 8-oxoG
OGG1	Bi (β)	8-oxoG, fapyG
NTHL1	Bi (β)	Tg, fapyG, DHU, 5-OHU, 5-OHC
NEIL1	Bi (βδ)	As NTH1 and fapyA, 8-oxoG
NEIL2	Bi (βδ)	Overlap with NTH1/NEIL1
NEIL3	?	Unknown
MPG	M	3-meA, hypoxanthine, ethanoA

*Type M= monofunctional; Bi=bifunctional

(Modified from Robertson *et al.*, 2009)

Thus, the primary function of glycosylases is to excise altered bases that cause only minor perturbations of the DNA double helix, releasing a free base and creating an apurinic or apyrimidinic site (AP, abasic site). Then, the DNA backbone is cleaved by either a DNA AP endonuclease (APE1), that generates 3'OH and 5'dRP (deoxyribose phosphate) termini or a DNA AP lyase, and in this case the 3' terminus at the cleavage site requires further processing, by APE1 or PNK, in order to provide a suitable substrate for a DNA ligase.

At this point, the DNA repair pathway could proceed via two sub-pathways depending on, among other factors, the state of the 5'dRP terminal moiety (Klungland and Lindahl, 1997): the short-patch (SP-BER), also named single nucleotide repair (SN-BER) and the long-patch (LP-BER).

In the SP-BER, the DNA polymerase β (Polβ), with an intrinsic dRPase activity (Prasad *et al.,* 1998), displaces the AP site and polymerizes DNA to fill in the gap, preparing the strand for ligation by a complex of DNA Ligase IIIα (Lig IIIα) and XRCC1 (X-ray repair cross-complementing group 1).

In the case where the 5' lesion is refractory to pol β lyase activity, the pathway switches to LP-BER (described for the first time by Dogliotti's group, Frosina *et al.*, 1996) in which polymerase δ, ε or β, coupled with PCNA (proliferating cell nuclear antigen), FEN1 endonuclease and Lig I synthesizes DNA to fill the gap and repairs the lesion.

The single strand break repair (SSBR) pathway utilizes many of the above mentioned BER proteins. Genotoxic agents that generate SSBs in the genome directly or indirectly activate PARP1 and PARP2.

PARP1 recognizes the single strand break and helps the recruitment of the repair machinery to the damaged site. The polymers generated by the activated PARP1 on DNA leads to the recruitment of the scaffold protein XRCC1, and then the gap-tailoring enzymes (polβ, Lig IIIα and PNKP) are recruited.

More recently, two additional minor sub-pathways of BER have been described: the Nucleotide Incision Repair (NIR), with a unique APE1-mediated repair initiation event that allows the removal of oxidized cytosine (Daviet *et al.*, 2007) and an APE1-independent BER sub-pathway for the repair of oxidized bases initiated by the bi-functional DNA glycosylases NEIL1 and NEIL2 (Wiederhold *et al.*, 2004; Das *et al.*, 2006).

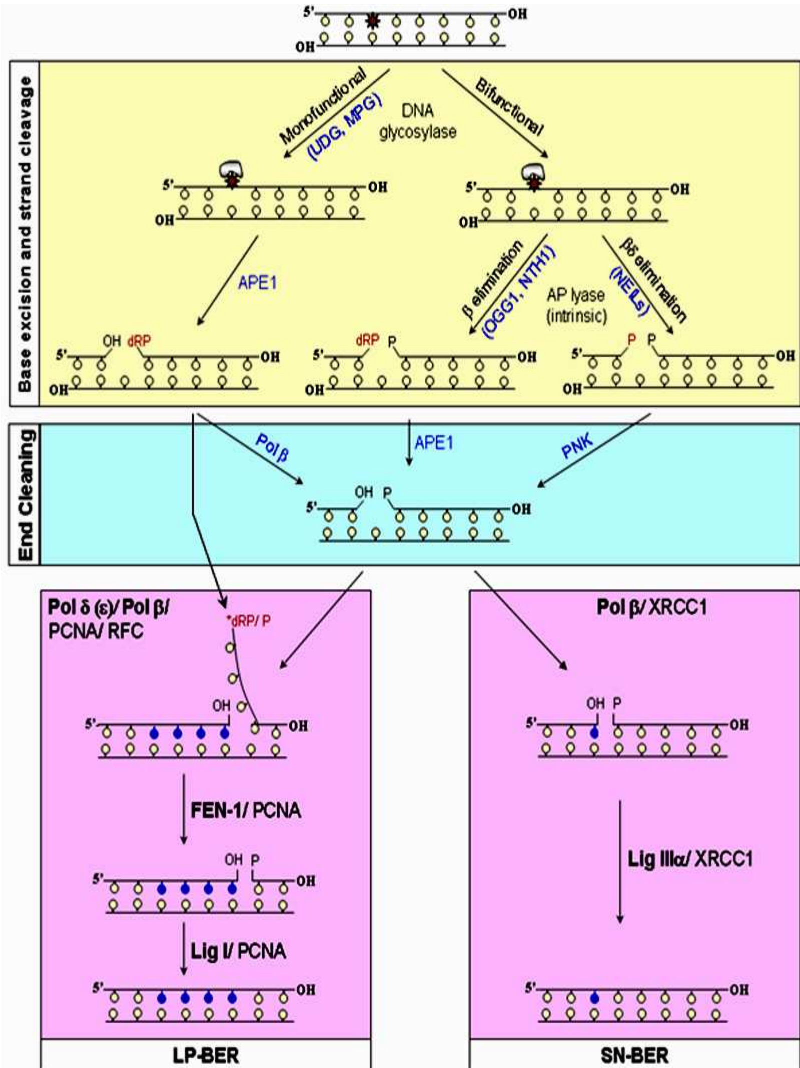

Fig.3 A schematic illustration of BER sub-pathways (LP- and SN- or SP-BER) for damaged bases and DNA single strand breaks. The damaged base is represented as a star(*). Divergent base excision steps converge to common steps for end processing, followed by repair DNA synthesis (represented as blue dots) and strand sealing. (From Hedge *et al.*, 2008)

1.2.1 XRCC1 (X-ray repair cross-complementing group 1)

Many of the genes involved in BER are highly conserved from bacteria to humans, indicating that this mechanism is a fundamental repair pathway in many living organisms. Even if no disease phenotypes have been linked to BER deficiencies, studies on knockout models have been improving our knowledge about BER protein functions.

Of particular interest is the case of *XRCC1* cloned in 1990; it was the first mammalian gene isolated which affects cellular sensitivity to ionizing radiation and is able to correct DNA strand break repair in the CHO-deficient cell line EM9 (Thompson *et al.*, 1990).

By the 1990s, XRCC1 was established as a BER scaffold protein: it has no enzymatic activity but acts coordinating primarily SP-BER and SSBR, and as described later, participating also in double strand breaks (DSBs) repair.

XRCC1 is one of the first proteins recruited to the nick generated by the action of a glycosylase, and to a single-strand break.

It has been demonstrated XRCC1 physically interacts with several components of BER, such as Lig III (Caldecott *et al.*, 1994), polβ (Kubota *et al.*, 1996), hOGG1 (Marsin *et al.*, 2003), APE1 (Vidal *et al.*, 2001), PNK (Whitehouse *et al.*, 2001) and PARP1 (El-Khamisy *et al.*, 2003) and activates many of them. (Figure 4)

These protein-protein partnerships can regulate pathway efficacy, dictating subsequent steps or sub-pathway choice. Furthermore, the process of the formation of complexes during the repair process appears to provide an increase in specificity and efficiency to the BER pathway. The regulation of BER is further refined through post-translational modifications, where the most common is phosphorylation. XRCC1 for instance is phosphorylated on at least four residues by CK2 (S518, T519, T523) and DNA-PK (S371) (Loizou *et al.*, 2004; Luo *et al.*, 2004; Levy *et al.*, 2006). (Fig. 4)

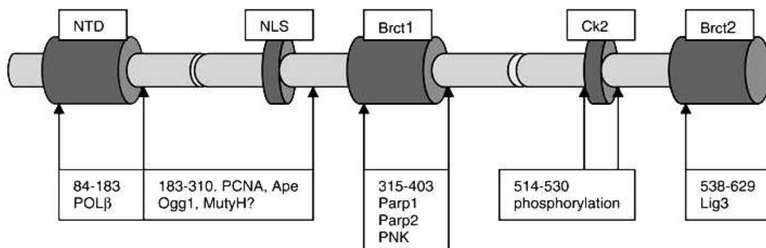

Fig.4 Human XRCC1 domains and locations of binding sites with interactive protein partners and of phosphorylation sites. (From Ladiges WC, 2006)

1.3 Nucleotide Excision Repair (NER)

NER is an extremely adaptable pathway that does not require a set of specific enzymes, each recognizing a different lesion but rather it senses the distortion caused by a damaged base and excises a tract of a few nucleotides around the lesion.

In this way NER repairs a broad-spectrum of damage caused by chemicals, that form bulky-adducts or by cross-linking agents and also by physical agents such as UV. This mechanism can ensures that active genes are rapidly repaired by the transcription-coupled repair (TCR) sub-pathway whilst the repair of non-coding and non-transcribed regions is carried out via global genome repair (GGR). A third sub-pathway, named DAR (transcription domain-associated repair) that operates on both strands in active genes has also been described (Nouspikel *et al.*, 2006; Nouspikel, 2009).

For the first two main sub-pathways the molecular mechanisms are well characterized (Figure 5); the first step is lesion sensing.

In mammalian GGR, the structural distortion is detected directly by the XPC-HR23B-centrin2 complex, which binds through XPC preferentially to the non-damaged strand. But in the case of a modest distortion the DDB complex, a heterodimer consisting of DDB1 and DDB2/XPE firstly recognizes the lesion and then recruits and stabilizes XPC by poli-ubiquitination.

In TCR the stalling of the RNA polymerase II at the lesion site in the transcribed strand actives genes and attracts the NER enzymes.

From this point, the two sub-pathways act using a common sequential mechanism. The next step involves the general transcription factor TFIIH, that possesses two helicase subunits (XPB and XPD) which form together with five other components, a ring-shaped structure that is able to open a denaturation bubble around the lesion. The TFIIH complex also recruits a DNA binding complex, made up of a XPA and RPA heterotrimer, that seems to displace the XPC complex (Hey *et al.*, 2002) and identifies the strand that carries the lesion (Sugasawa *et al.*, 1998).

The damaged strand is then incised by the XPG endonuclease and the XPF-ERCC1 complex, on the 3' and the 5' side respectively; in mammals the fragment excised is about 25-30 nucleotide in length. The two final steps consist of filling in the resulting gap by either DNA polymerase δ or ε, probably associated with PCNA, and in sealing the nick by ligase III, together with XRCC1. In actively replicating cells, ligase I concludes the NER process. (Moser *et al.*, 2007)

In contrast to the situation for BER genes, several genetic diseases have been described to be caused by mutations in genes involved both in GGR

and TCR, such as Xeroderma pigmentosum (XP), Cockayne syndrome (CS). But as seen for the BER proteins, post-translational modifications and protein-protein interactions can further regulate NER.

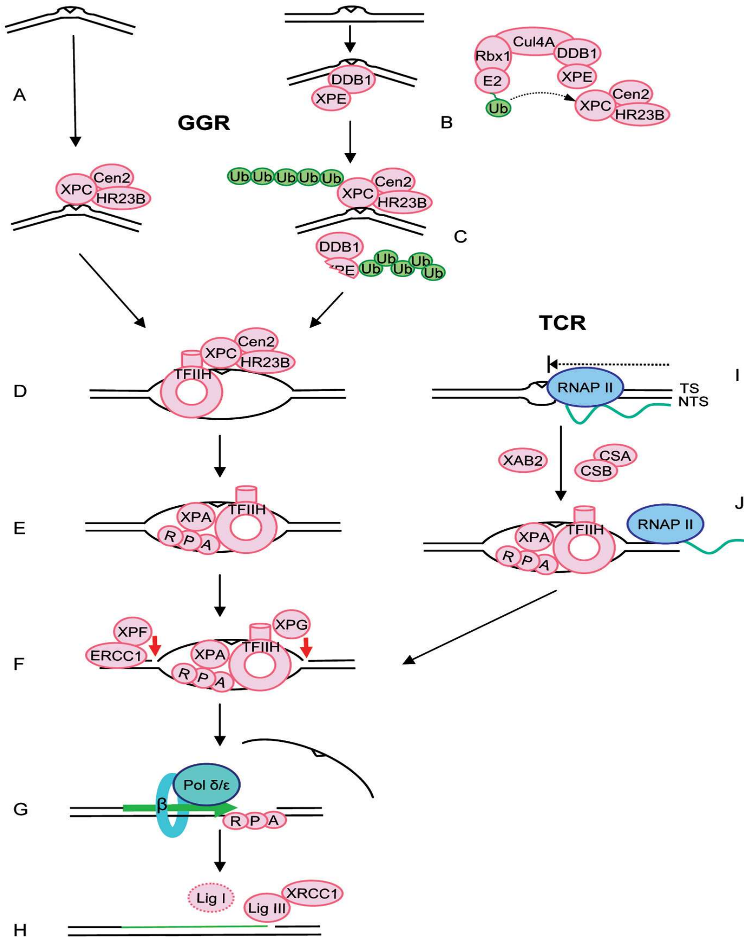

Fig.5 Mechanism of NER. DNA lesions can be detected directly by XPC-HR23B-Cen2 (A) in GGR or by DDB complex (B) in TCR. After differences in the first step of lesion sensing, the two sub-pathways share a common mechanism which is detailed in the text. (From Nouspikel, 2009)

1.4 Double Strand Breaks repair: NHEJ and HRR

As shown previously, in BER and NER mechanisms single strand breaks (SSBs) are an enzymatic consequence of the repair of DNA damaged bases but they can represent a serious risk for cells if they are not filled by a polymerase and rejoined by a DNA ligase. In fact, during DNA replication SSBs can be converted to more lethal DNA DBSs.

Apart from the collapse of replication forks when the replication machinery encounters SSBs, DSBs can arise directly from exposure to ionizing radiation (IR) and radiomimetic chemicals, and in a programmed manner during meiosis and immunoglobulin gene rearrangements, as simplified in Figure 6.

DSBs are regarded as the most toxic of DNA lesions because, in addition to the potential of causing mutations, they could lead to deletions, chromosome translocations, genomic instability (Elliott and Jasin 2002; Thompson and Schild, 2002) and under some circumstances they can induce cell cycle arrest and apoptosis (Norbury and Hickson, 2001; Jackson, 2001). (Fig.6)

Homologous Recombination Repair (HRR) and non-homologous end joining (NHEJ) are the two major pathways of DSBs repair. A third system, a nonconservative process named single-strand annealing (SSA), shares HRR and NHEJ components.

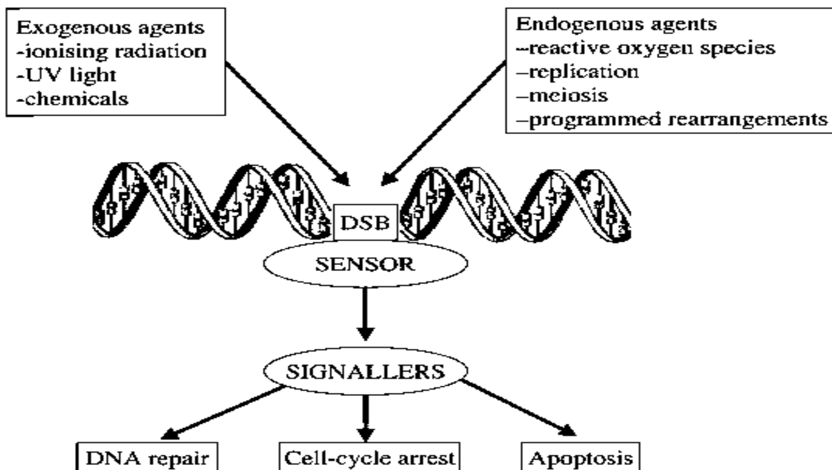

Fig.6 Simplified scheme of exogenous and endogenous agents causing DSBs and consequently possible cell responses. (From Jackson, 2001)

The fundamental difference between HRR and NHEJ is the dependence on DNA homology. HRR takes place in cells that have already replicated so it is an error-free mechanism specific to late S and G_2 cell cycle phases; on the other hand, NHEJ is an error-prone pathway, predominant in higher eukaryotes, that occurs in early S and G_0/G_1 cells that lack a homologous template.
In NHEJ whenever a DSB is generated, the DNA ends are bound by the Ku heterodimer (Ku70 and Ku80) together with, in mammals, the DNA-dependent protein kinase (DNA-PK). This stimulates DNA-PK kinase activity and it can subsequently phosphorylate a variety of DNA-binding proteins thus regulating temporally the sequence of events in this pathway. Among the possible targets are KU, XRCC4/Ligase IV and also itself: this automodification leads to its dissociation from DNA (Lees-Miller *et al.*, 1990; Chan and Lees-Miller, 1996; Leber *et al.*, 1998). Additionally, the XRCC1 scaffold protein that acts mostly in BER mechanism can be a further substrate for DNA-PKcs. This phosphorylation on Ser 371 results in its involvement in DSBs repair. (Lévy *et al.*, 2006)
The XRCC4/Lig IV complex is a crucial component of NHEJ: it mediates the final DNA strand-joining step. Occasionally, a direct end-ligation may be possible but in most cases DSBs require processing before ligation.
For radiation-induced DSBs, as described in Valerie and Povirk's (2003) model resumed in Figure 7, the protruding 3' termini are processed by the combined action of two enzymes: TDP1 and PNKP.
A complex of Ku, XRCC4/Lig IV and a DNA polymerase, probably Polμ (Mahajan *et al.*, 2002), then forms at the end-to-end junction and catalyzes gap-filling and ligation, completing the repair process.
It is evident that this mechanism not requiring an undamaged partner molecule could lead to errors and thereby mutations. Furthermore, sequence deletions may be introduced by action of the MRE complex (Rad50-Mre11-NBS1 in mammals) which possesses nucleolytic activity.
The Rad50-Mre11-NBS1 complex is also involved in the early step of the HRR pathway (Figure 8) of DSBs recognition and resection through MRE's 5'→3' nuclease activity to yield single strand overhangs.
ATM could control this step since NBS1 is a direct substrate for ATM phosphorylation on at least three different sites. (Lim *et al.*, 2000) Moreover, ATM is able to phosphorylates histone H2AX at Ser 139, and this occurs rapidly (1-3min) in response to ionizing radiation, leading to an "opening-up" of the chromatin and foci formation. (Rogakou *et al.*, 1998; Downs *et al.*, 2000).

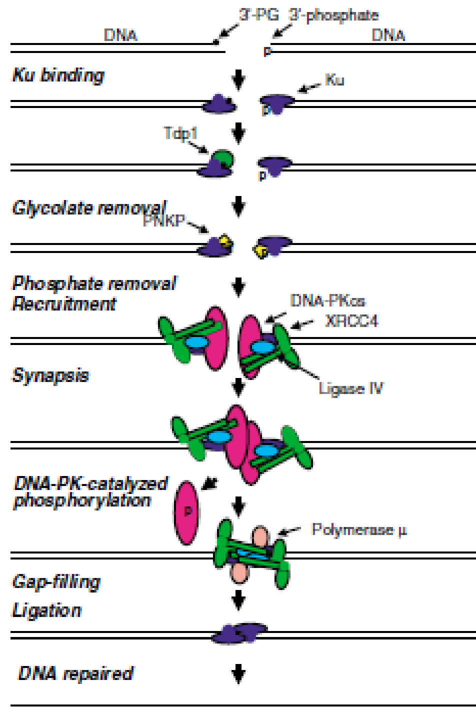

Fig.7 Model to repair radiation-induced DSBs through NHEJ, proposed by Valerie and Povirk (2003).

After nuclease action, the 3' single stranded DNA tails are bound by RAD51 that forms nucleoprotein complexes, coated by RPA, to mediate a search for homologous sequence on an undamaged partner molecule and, when these have been found, it catalyses a strand exchange reaction.
The RAD51 paralogs, RAD52 and RAD54 seem to facilitate the action of RAD51 binding. (Tan *et al.,* 1999). Recently, it was demonstrated that also BRCA2 (breast cancer susceptibility protein) helps loading of RAD51 onto the DNA. (Yang *et al.*, 2002)
XRCC3, a member of the recA-like gene family, functions as an accessory strand transfer protein whose function is not limited to HR initiation but extends to late stages in formation and resolution of HR intermediates, possibly by stablilizing heteroduplex DNA. (Brenneman *et al.*, 2002)

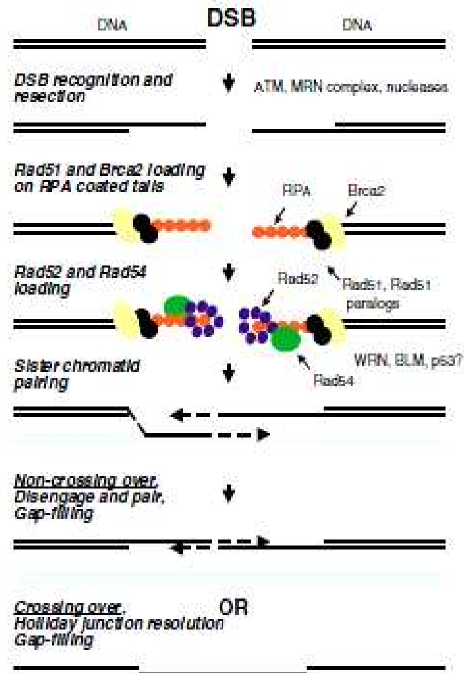

Fig.8 The steps in HRR. In the earliest stages, ATM senses the DSBs and phosphorylates H2AX, which would then attract BRCA1 and NBS1. BRCA1 coordinates the repair events that follow and the MNR complex resects the DNA to provide ssDNA overhangs. BRCA2, attracted to the DBS by BRCA1, facilitates the loading of RAD51 with the help of RAD51 parologs and of the BLM and/or WRN helicases. The tumor suppressor p53, known to interact with BRCA1, RAD51, BLM and WRN is also found in this DNA-protein complex. From this point, there are two possibilities to finish the repair process: either by non-crossing over or by crossing-over. (From Valerie and Povirk, 2003)

Following sister-chromatid pairing and strand invasion of DNA overhangs, HRR can go on in two directions: the non-crossing over path in which the Holliday junction is disengaged, the gap in the damaged strand is filled in by DNA polymerase and the ends are then sealed by Ligase I; or the classical crossing over path, in which after DNA polymerase synthesis and Lig I sealing, the Holliday junctions have migrated and resolved to yield two intact DNA molecules. As a consequence, HR generally leads to accurate, error-free repair. In mammals, two helicases encoded by the *BLM* and *WRN* genes, associated with Bloom's syndrome (BS) and Werner's

syndrome (WS) respectively, interact with the Holliday junctions and play an important role in this pathway (Constantinou *et al.*, 2000; Mohaghegh *et al.*, 2001).

Considering the severity of DSBs, the above described pathways collaborate in the cell to maintain genomic stability and to repair a double strand break with minimal error. In fact, it has been suggested that when one of them is impaired, the other seems to act to compensate its deficiency (Pluth *et al.*, 2001; Allen *et al.*, 2002).

Moreover, NHEJ seems to be helped by some components of the BER pathway in repairing DSBs; Audebert *et al.*, (2004) demonstrated that DNA-PK, XRCC4/Lig IV complex interact also with PARP1 and XRCC1/Lig III to approach, firstly, DNA ends and then to seal them.

In addition to crosstalk and coordination between different types of DSBs repair pathways, there is also a balance between pro- and anti-apoptotic mechanisms that potentially modulate repair (Bernstein *et al.*, 2002). The DNA damage sensors, especially ATM, are able to phosphorylate, at different sites, the tumour suppressor gene p53. p53 acts as a transcriptional factor; it could control cellular destiny by activating cell cycle check-points, thus promoting firstly DNA repair, or alternatively apoptosis (Offer *et al.*, 2002) to avoid the accumulation of an excess of DNA damage which may lead to carcinogenesis. The different processes controlled by p53 are resumed in Figure 9.

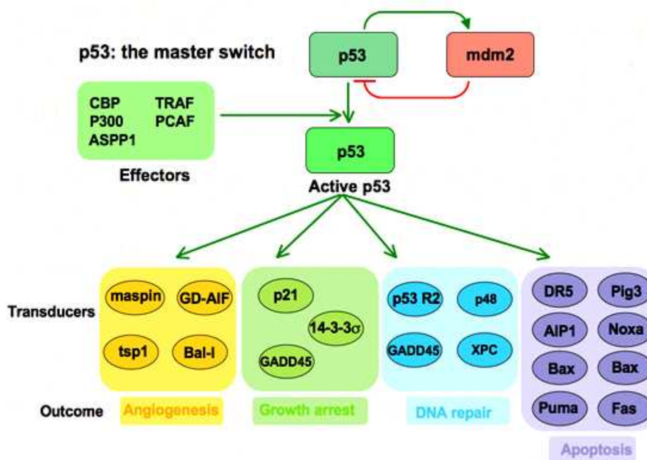

Fig.9 p53 downstream events and final cellular outcome. (http://www.p53free.fr)

2. Gene polymorphisms and individual susceptibility

It is well known that defects in DNA repair mechanisms caused by mutations in DNA repair genes, as described in the previous section, are associated with human disorders.

Still, despite the lack of a marked pathological phenotype, humans carrying variants in DNA repair genes could show an impaired DNA repair capacity and a different response to DNA damage that could be associated for instance with an increased sensitivity to DNA damaging agents. Furthermore, related to an impaired DNA repair capacity and an increased mutagenesis, variants in DNA repair genes could also modify the risk of developing cancer.

The principal source of inter-individual variability is represented by genetic polymorphisms which are allelic variants at a genetic *locus* that have a frequency higher than 1% at the population level. They can be summarised into three different classes: the *Single Nucleotide Polymorphisms* (SNPs), deletions or insertions in DNA sequence, and *Variable Number of Tandem Repeats* (VNTR).

About 1.4 million of SNPs have been described by the Human Genome Project and 93% of human genes have almost one SNP but their distribution is not uniform throughout the whole genome. They can be present in exonic, non-coding, such as 5'UTR and 3'UTR, or regulatory regions, thus altering the structure of encoded proteins, levels of protein expression or mRNA stability by for instance modifying the affinity of microRNAs (Shastry, 2009).

In the last few years these small-non coding RNAs, that act through a post-transcriptional mechanism targeting the complementary mRNA target, have been proposed to be related to cancer risk (Grønbaek *et al.*, 2007; Iorio and Croce, 2009).

When present in exonic regions, SNPs can also invoke a change in amino-acid (AA) sequence, too. If the replaced AA is the same as the original one, the SNP is called synonymous. On the other hand, a non-synonymous SNP gives rise to an AA substitution that may alter protein function and for this reason their effects are the object of many research investigations.

SNPs have been reported in members of the BER pathway. As described above the human *XRCC1*, located on chromosome 19q13.2, encodes for a 633aa protein which coordinates the recruitment of other BER components, such as polymerase β, APE1, hOGG1, PARP and ligase III through NH2-terminal, BRCT I, and BRCT II, COOH-terminal domain respectively, as shown in Figure 10.

In 1998 Shen and co-workers (Shen *et al.*, 1998a) described three polymorphisms in the *XRCC1* gene, which resulted in non-conservative

amino-acid changes in evolutionary conserved regions: the C→T substitution in codon 194 of exon 6 (Arg to Trp) [rs1799782]; the G→A substitution in codon 280 of exon 9 (Arg to His) [rs25489] and the G→A substitution in codon 399 of exon 10 (Arg to Gln) [rs25487]. (Fig. 10)
Recently, Hao and colleagues (2004) identified, in a Chinese population, another variant in the *XRCC1* gene located in the 5'UTR (5'-untraslated region), -77T→C (Fig. 10). This polymorphism has been also confirmed to be present, with a high frequency, in Caucasian populations (Brem *et al.*, 2006; Sterpone *et al.*, 2010b)
Whilst the three exonic SNPs could impair protein-protein interactions and therefore XRCC1 repair capacity (Lunn *et al.*, 1999), the promoter T/C substitution might disrupt a consensus sequence for Sp1-binding site (Hao *et al.,* 2006), so that this polymorphism may have the potential to alter *XRCC1*'s transcriptional level.

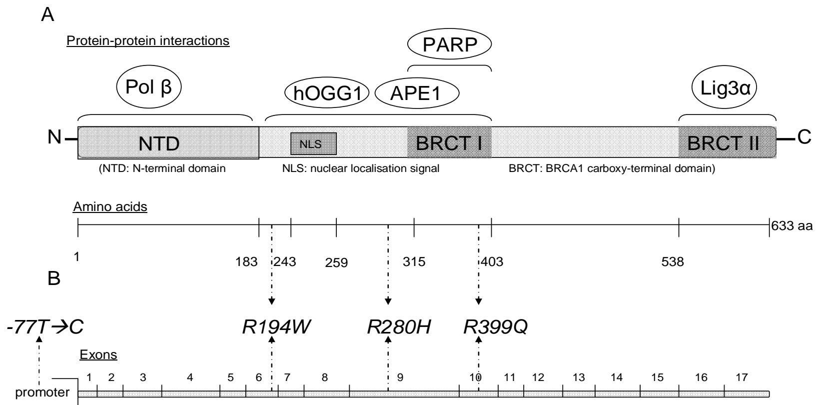

Fig.10 Human XRCC1 protein and gene structure. A: the diagram shows XRCC1 domains and the regions of interaction with other components of BER; B: the diagram shows the structure of the gene with the most common and studied single nucleotide polymorphisms (SNPs): -77T→C, R194W, R280H and R399Q. Each of them is detailed in the text. (From Sterpone and Cozzi, 2010)

Several SNPs giving rise to amino-acid substitutions have also been described in the *OGG1* gene, of which the Ser326Cys in exon 6 (rs1052133) is the most common. This polymorphic amino-acid is located outside the domains conserved among DNA glycosylases. The functional differences between the two polymorphic OGG1 proteins in human cells

still remain unclear; Yamane *et al.* (2004) and Bravard *et al.* (2009) suggested that *OGG1*-Cys326 protein had a reduced 8-oxoG DNA glycosylase activity and therefore a lower ability to suppress mutation than the wild-type protein.

In the *XRCC3* gene, whose encoded protein is a member of the family of Rad 51-like proteins (Liu *et al.*, 1998) that participates in homologous recombination, sixteen allelic variants have been reported. The most studied substitution Thr to Met in codon 241 (due to a transition C>T in exon 8) [rs861539] does not reside in the ATP-binding domains, which are the only functional domains identified in the protein (Shen *et al.*, 1998a). Concerning the recombinase RAD51, to date fourteen SNPs have been described and two of them are within non-coding regions: they are named *RAD51-01* (rs1801320) and *RAD51-52* (rs11855560). The former in the 5'UTR seems to modify the alternative splicing of *RAD51* transcripts and the penetrance of *BRCA1/2* mutations (Antoniou *et al.*, 2007), and the latter is in the 3'UTR which is thought to be a microRNA binding site from a bioinformatic analysis.

For several years there has been an interest in assessing possible links between genetic factors, in particular low penetrance variants such as SNPs, and increased/decreased risk of tumour.

To this day, molecular epidemiological studies have shown that a number of links exist; for instance *XRCC3*-Thr241Met polymorphism has been implicated in an increased risk of melanoma (Winsey *et al.*, 2000); SNPs in *XRCC1* seem to be associated with several kinds of cancers, such as lung (Divine *et al.*, 2001; Chen *et al.*, 2002) and pancreatic adenocarcinoma (Duell *et al.*, 2002), oral carcinoma (Hsieh *et al.*, 2003; Hao *et al.*, 2004) as well as bladder (Matullo *et al.*, 2001; Shen *et al.*, 2003) and breast cancer (Huang *et al.*, 2009). *RAD51-01* SNP is also thought to be associated with breast cancer risk, especially in view of the links with BRCA (Gao *et al.*, 2010; Zhou *et al.*, 2010). Case-control studies on the SNP in codon 326 of *hOGG1* (8-oxoG-DNAglycosylase) suggest that this polymorphism may be a risk factor for lung adenocarcinoma (Ito *et al.*, 2002; Hung *et al.*, 2005) and stomach cancer (Takezaki *et al.*, 2002), but the 326Cys allele plays a protective effect to breast cancer in European women (Yuan *et al.*, 2010).

Since studies have given conflicting results in some cases due to ethnicity, small number of samples and mainly to low statistical power, the relationship between genetic polymorphisms and cancer occurrence is not so clear. Hence, in this interesting field of research further investigations are required to better understand the influence of the individual genetic background in order to select SNPs as possible genetic markers for cancer susceptibility.

3. Radiosensitivity

Other than cancer susceptibility, the individual's allelic architecture (Andreassen *et al.*, 2003), or combination of SNPs in different genes that are carried, and of particular relevance are genes involved in DNA repair processes, may influence responses to ionizing radiation (IR), a physical agent used in radiotherapy (RT). RT is an efficient and widely used modality for cancer treatment due to the characteristics of IR to produce a variety of DNA lesions.

Radiation extends its effects on DNA by direct interactions (van der Schans *et al.*, 1973) and by radiolysis of H_2O that generates oxygen radical species capable of causing indirect damage to DNA (von Sonntag, 1987). Hence, radiation exposure activates DNA signalling and repair pathways to correct both SSBs and DSBs which have been generated in both normal and cancer cells, without exception.

Interestingly, cancer patients receiving radiotherapy display a great inter-individual variability in their responses to IR exposure and the risk of developing adverse reactions in normal tissues could be different among them. The term adverse reactions include acute effects, such as erythema, desquamation, dermatitis, as well as late effects that can develop months or years later, such as telangectasia, fibrosis and rarely, secondary tumours.

Although part of this inter-patients variability can be ascribed to differences in treatment as well as in fraction size and tumour characteristics, it has become clear that there might be an important genetic component to explain radiosensitivity (Andreassen *et al.*, 2002; Fernet and Hall, 2004; Andreassen, 2005).

New insights into the underlying molecular mechanisms of this sensitivity are coming from radiogenomics studies that assess possible associations between common genetic variants, mostly in DNA damage detection and repair genes, and the development of adverse reactions to radiotherapy. Recently, a Radiogenomics Consortium was established in United Kingdom with the aim of collecting such studies conducted throughout the world in order to perform meta-analysis and achieve more significant conclusions (West *et al.*, 2010). The goal of radiobiology is to identify genetic markers and to develop *in vitro* tests which allow to predict clinical radiosensitivity in order to adjust RT protocol for both radiosensitive and radioresistant patients. (Figure 11)

To date, as resumed by Jeggo and Lavin (2009) the most frequently used techniques to monitor SSBs and DSBs formation and repair, and thus evaluating cellular radiosensitivity, are sucrose gradient sedimentation; alkaline and neutral elution (Iliakis *et al.*, 1991a); pulsed field gel electrophoresis (Iliakis *et al.*, 1991b), alkaline and neutral comet assay

(Singh *et al.*, 1989; Olive *et al.*, 1991) and H2AX foci formation. Moreover, studies on patients with radiation sensitive disorders (i.e. Ataxia Telangiectasia AT; AT like Disorder ATLD; Nijmegen Breakage Syndrome NBS) and radiation sensitive mutant models (i.e. the rodent *XRCC1*-deficient CHO cell line, EM9) have been providing important insights into the identification of genes critical for radiation resistance.

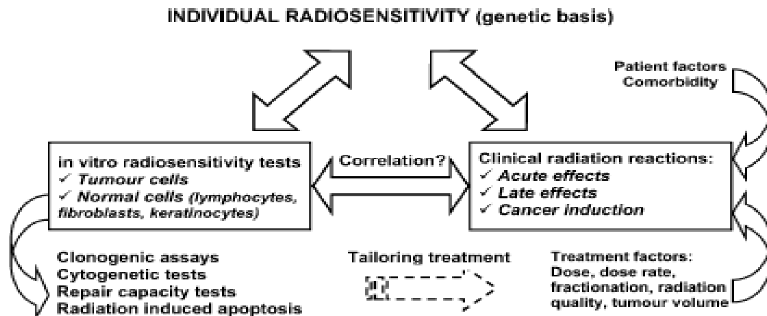

Fig.11 Relation between clinical and *in vitro* radiosensitivity, considering factors that influence side-effects. The way forward lies on the development of predictive assays which allow tailoring patient's treatment. (From Bourguignon *et al.*, 2005)

3.1 XRCC1 and radiation exposure

In the 1980s Thomson *et al.* (1980; 1982) described a Chinese Hamster Ovary (CHO) cell strain characterized by hypersensitivity to (m)ethylation agents, sensitivity to ionizing radiation, accumulation of single strand breaks in DNA after damage and an unusually high frequency of sister chromatid exchanges (SCEs). This cell line, denoted EM9 was isolated from mutagenised AA8 cell line and was found to be deficient in the XRCC1 protein, and in LigIII activity (Caldecott *et al.*, 1995).

A few years later, Shen and co-workers (1998b) described the mutation in the hamster *XRCC1* coding sequence found in EM9 cells. The EM9 cells are capable of expressing the *XRCC1* gene, but the protein is absent as result of a C→T substitution at nucleotide 661 that introduces a termination codon. Consequently a truncated polypeptide (220 residues) lacking two thirds of the normal sequence is produced. This result means EM9 line is effectively a null mutant that provides a model to understand functional role of XRCC1. Therefore, many studies have been conducted using the EM9 cells to investigate the biological consequences of a XRCC1 deficiency. To date, it is known the lack of XRCC1 is responsible for sensitivity to DNA-

damaging agents like IR, reduced rate of single (SSBR) and double strand break repair (DSBR) and perturbation of DNA replication. (Dillehay *et al.*, 1983 ; Schwartz *et al.*, 1987; Thomson *et al.*, 1990).
Furthermore, by using RNA interference technique Brem and Hall (2005) clearly demonstrated that *XRCC1* is required for efficient SSBR and genomic stability in human cells. Overall these findings suggest that *XRCC1* is an interesting genetic factor which, both in its wild type and polymorphic forms, can play an important role in determining the radiosensitivity.
Beyond investigations at the protein level, another important question is whether *XRCC1* gene expression could correlate with differential radiosensitivity. Although a low-abundance of *XRCC1* mRNA was found in rat, mice and most mammalian tissues (Yoo *et al.*, 1992; Walter *et al.*, 1994; Zhou and Walter, 1995; Thomson and West, 2000), exposure to a wide range of DNA damaging agents, including IR, has not shown any convincing changes in *XRCC1* mRNA levels (Thomson *et al.*, 1993). However, transcription of *XRCC1* was reported to be enhanced by irradiation in ataxia telangectasia cells (Shung *et al.*, 1994) and a radioresistant mouse showed higher levels of *XRCC1* mRNA (Labudova *et al.*, 1997a; Labudova *et al.*, 1997b). So, the *XRCC1* mRNA inducibility could represent an additional interesting field of research, especially in the light of recently developed techniques such as quantitative RT-PCR that are more sensitive than techniques such as northern blotting.
Radiosensitivity is considered to be a polygenic trait which also depends on the cell cycle. Generally, mammalian cells are more radiosensitive during G_2 and M than S phase (Sinclair and Morton, 1966) and this can in part be explained in terms of DNA repair efficiency.
For single strand break repair, Caldecott noted (2003) that cells possess a rapid XRCC1-dependent process that operates throughout the cell cycle (global rapid SSBR) and by which SSBs are rejoined within 0.5-3h, but also a S/G_2 specific XRCC1-dependent pathway. This latter allows repair of single breaks induced specifically in S phase but also those persisting from G_1, in order to prevent DSBs formation and fork collapse. (Figure 12)
BRCT domains of XRCC1 have been demonstrated by Taylor's group to have a cell cycle-specific role. In fact, global rapid SSBR is inhibited by mutations in BRCT II which disrupt XRCC1-Lig IIIα interaction (Taylor *et al.*, 2000), whereas S-phase SSBR is ablated by mutations within BRCT I (Taylor *et al.*, 2002). The BRCT I domain is of fundamental importance in linking the SSBR machinery and the replicative apparatus, as Lévy and colleagues (2009) reported; in fact, it interacts with the p58 subunit of DNA Polα-primase complex, which begins DNA replication by RNA primer

synthesis, and this interaction helps to stabilize stalled fork preventing collapse. In the case of damage to DNA, XRCC1-BRCT I stimulates PARP1 so that primase activity is inhibited by poly(ADP)ryboses and thus cells have more time to repair lesions prior to resuming the replication process. The inhibition of DNA primase is facilitated by the interaction of XRCC1 with p58 subunit (Lévy *et al.*, 2009).

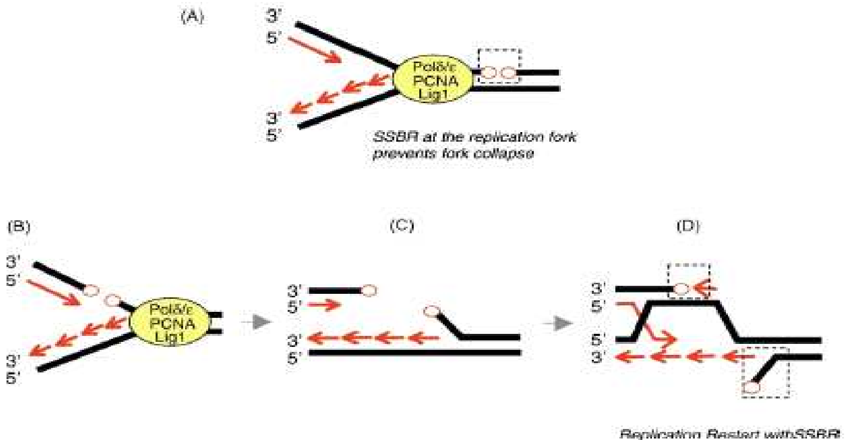

Fig.12 A model for S/G2-specific XRCC1-dependent SSBR. An unrepaired SSB in the template (black) strand is encountered by the DNA replication machinery (yellow oval) and is repaired ahead of the fork (A). Alternatively, the SSB collapses the replication fork and creates a DSB (B and C). In this case, homologous recombination reactions result in a Holliday junction and restoration of the replication fork (D). (From Caldecott, 2003)

Such a specific recruitment of XRCC1 protein during the replicative phase of the cell cycle seems to correlate positively with data in the literature that show an induction of *XRCC1* expression by transcription factors generally important in the regulation of S-phase specific genes and progression into DNA replication.

In response to DNA damage, for instance, the two transcription factors FOXM1 and E2F1 are phosphorylated and their activated forms are able to bind directly to the *XRCC1* promoter thus increasing *XRCC1* transcription (Tan *et al.*, 2007; Chen *et al.*, 2008).

Adequate levels of XRCC1 are important to allow DNA repair and once it has occurred, to continue cell progression. In fact, as Brem and Hall (2005)

showed, RNA interference lowered levels of XRCC1 protein lead to a significant delay in S-phase after MMS treatment.
So, as radiosensitivity is a complex trait dependent on many factors studies have to be conducted at different levels to understand the complicated cellular mechanisms of the responses to IR exposure.

II. AIM of the RESEARCH

The aim of this research was to analyse the relationship between induced DNA damage, the DNA damage responses and the genetic background. We concentrated on studying the influence of variant alleles in damage signalling and repair genes on individual susceptibility to developing cancer and on sensitivity to IR-exposure, analysed both *in vivo/ex vivo* and using *in vitro* systems.

Ex vivo studies were focused on cancer patients, enrolled in Italian and French Oncology Units. Radio-induced DNA damage and its repair play a critical role in the susceptibility of patients to develop side effects after radiotherapy (RT). Therefore, the development of *in vitro* cellular radiosensitivity tests and genetic markers, that can be used as biomarkers for the extent of patients' normal tissue reactions, is of great interest. Such markers could be used to adjust RT protocols for both radio-sensitive and -resistant patients.

In the first part of this project, we investigated the *in vitro* capacity to repair ionizing radiation induced damage in peripheral blood leucocytes (PBLs) of Italian sporadic breast cancer (BC) patients, who had not yet had RT, in relation to their genotype. Blood samples was irradiated *in vitro* with 2 Gy of X-rays and the DNA primary damage (namely single strand breaks and double strand breaks) and the repair kinetics, at defined time intervals after irradiation (0, 30 and 60 min), were measured using the alkaline Comet assay. All patients and matched healthy controls were genotyped for SNPs in a panel of repair genes involved mainly in two different repair pathways: Base Excision (BER) and Homologous Recombination (HRR) Repair. Through RFLP-PCR, we defined the genotypes for *XRCC1* (taking into account three allelic variants in *XRCC1*: -77T→C in the promoter, Arg194Trp and Arg399Gln), *OGG1* (Ser326Cys) and *XRCC3* (Trp241Met). The induced DNA damage and the repair capacity were then correlated with each genotype in order to investigate whether this could provide a possible explanation for the observed inter-individual responses to X-ray treatment.
Additionally, the correlation between the adverse healthy/normal tissue side effects (graded by S.Camillo's clinicians using the Radiation Therapy Oncology Unit score) and the individual genetic background was analysed in a cohort of Italian BC patients who had previously had radiotherapy.

Then, since it has been reported that the presence of genetic variants in multiple repair pathways may have a joint or additive effect on BC risk and on development of RT adverse reactions, we examined the association of the 5 SNPs, and in particular *XRCC1* haplotypes, with the risk of sporadic breast cancer and the radiotherapy-induced early adverse reactions in Italian BC patients.
During my PhD I also spent six months (from January to June 2009) at the INSERM U612 based at Institut Curie (Orsay, France) to study the influence of polymorphisms in *RAD51*, a key player in the DNA double strand breaks response, on individual radiosensitivity. The aim was to investigate whether two SNPs in the DNA repair gene *RAD51*, ex10-52T>C (*RAD51-52*) located in a miR34a* miRNA binding site and ex1-96G>C (135G>C: *RAD51-01*), that seems to modify *BRCA1-BRCA2* mutation penetrance, were associated with an increased risk of developing cancer and adverse reactions to radiotherapy in two cohorts of breast cancer patients, established from Italian and French Hospitals.
In addition, lymphoblastoid cell lines (LCLs), established from patients homozygous for wt and variant *RAD51-52* alleles, was screened to assess whether the expression of miRNA34a* and the SNP genotypes might influence RAD51 expression and *in vitro* cellular responses to radiation.
We first genotyped all the BC patients and healthy controls for the *RAD51-01* and *RAD51-52* SNPs using RFLP-PCR and sequencing, respectively. Then to assess if *RAD51-52* polymorphism could influence the level of *RAD51* mRNA or miR34a* binding, as theoretical bioinformatic analysis suggested, we determined basal levels and levels after treatment with ionizing radiation (5Gy of γ-rays) and recovery at different time points (0, 2, 4, 8, 24h) of *RAD51* and *p21* mRNA and miR34a* in the LCLs with different *RAD51* genotypes. The analyses were performed by RT-qPCR using the TaqMan gene expression assay-comparative Ct method, which allows the quantitative expression of a cDNA target, normalized with an endogenous control and a reference sample. Moreover, we assessed the influence of the *RAD51-52* polymorphism at the protein level, by determining basal levels and levels after the treatment with IR of RAD51 protein levels and in parallel the p53 protein levels by western blotting in the same cell lines.

In order to better understand the role of one gene already characterized in my lab at the Department of Biology in Rome, *XRCC1*, we analysed the biological responses to X-ray treatment in two hamster cell lines derived from CHO (Chinese Hamster Ovary): the EM9 null XRCC1 mutant, as

described in above (pg.34), and the wild type parental cell line, AA8, as a control.

By comparing in these two cell lines the response to DNA damage, we assessed whether the lack of functional XRCC1 could influence: a) the stability of *XRCC1* mRNA; b) the *XRCC1* gene expression measured through quantitative real time reverse-transcriptase PCR (RT-qPCR with SYBR Green I); c) the *XRCC1* expression in the different phases of the cell cycle by RT-qPCR and finally d) the protein expression valued by Western blotting.

In the final part of this project we focused on studying in depth the mechanism of Base Excision repair (BER), in which XRCC1 is mainly involved using quiescent peripheral blood mononuclear cells (PBMCs). To do this, we analysed the time course of *XRCC1* mRNA expression and the repair kinetics after IR exposure in G_0 PBMCs isolated from buffy coat of healthy donors, obtained from S. Camillo Forlanini Hospital-Rome.

After the isolation, performed using Lympholyte gradient separation medium, the quiescent peripheral blood mononuclear cells were irradiated with 2Gy of X-rays and at different time points (0, 15, 30, 60, 90 and 120 min) total RNA was extracted and radio-induced DNA damage repair was measured through alkaline Comet assay.

III. RESULTS

1. Genetic polymorphisms, repair capacity and breast cancer risk

In peripheral blood leucocytes (PBLs) of 35 BC patients, not yet submitted to radiotherapy, and 34 age- and sex-matched healthy controls we studied cellular response to X-ray exposure using the alkaline Comet assay.
The characteristics of the studied populations are resumed in Table 1.

Table 1 Characteristics of populations involved in the study

	Breast Cancer Patients	Healthy Controls
All	35	34
Current age (years±SD)	58±10.5	52±9.4
Age (range)	40-76	40-70
Smokers	8 (23%)	9 (26%)
Not smokers	22 (77%)	25 (74%)
RTOG/EORTC* score of early adverse effects (Organ tissue: skin)		
G0	15 (43%)	
G1	14 (40%)	
G2/G3	6 (17%)	

* RTOG/EORTC [8]: Radiation Therapy Oncology Group/European Organization for Research and Treatment of Cancer scale. G0 (grade 0): no change over baseline; G1 (grade 1): follicular, faint or dull erythema/epilation/dry desquamation/decreased sweating; G2/G3 (grade 2/grade 3): tender or bright erythema or moderate edema/confluent, moist desquamation other than skin folds, pitting edema.

(Modified from Sterpone *et al.*, 2010a)

In Figure 1 we showed the basal level and the X-ray induced DNA damage measured as % of DNA in the Tail (TD). The BC patients exhibited a very significantly ($p<0.001$) higher mean level of basal DNA damage when compared to healthy controls (1.6 vs 0.83). Immediately after irradiation and 30 min later BC patients had significantly higher ($p<0.01$) level of DNA damage and this level persisted significantly ($p<0.05$) higher 60 min later, in comparison to healthy subjects.
In terms of the kinetics of DNA repair, in both groups DNA damage slowly decreased 30 and 60 min after irradiation, never reaching the basal level. In BC patients we observed a significantly higher value of DNA damage both after 30 and 60 min when compared to basal levels (3.7 and 2.5 vs 1.6, $p<0.0001$). In healthy subjects a similar trend was observed with a less significant difference (2.5 and 1.7 vs 0.83, $p=0.008$ and 0.002, respectively). When residual damage (RD) was considered, we found 55% and 26% of

RD in BC patients at 30 and 60 min, respectively and 40% and 28% of RD in controls at the same times (data not shown in figure).

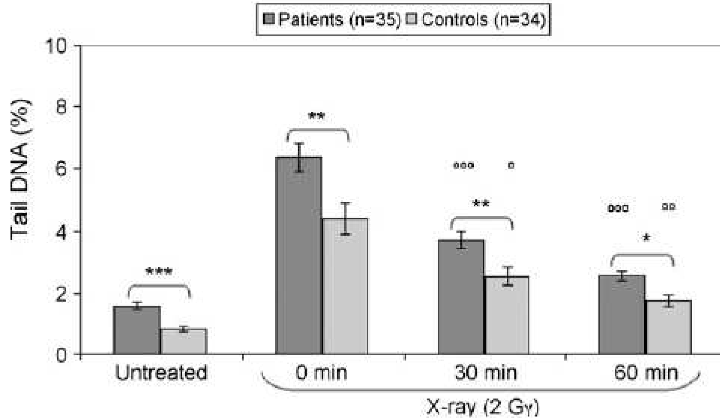

Fig.1 Basal and X-ray induced DNA damage expressed as Tail DNA in BC patients and controls. The Comet assay was performed in untreated condition (0Gy), immediately after irradiation (2Gy) and 30 and 60 min later. ***p< 0.001, **p< 0.01, *p<0.05 at Mann–Whitney U-test when comparing BC patients to controls; °°°p<0.0001 at ANOVA nonparametric Friedman test when comparing TD values at 30’ and 60’ after irradiation with basal value in BC patients; °p=0.008, °°p=0.002 at ANOVA nonparametric Friedman test when comparing TD values at 30’ and 60’ after irradiation, respectively with basal value in controls. (Taken from Sterpone *et al.*, 2010a)

Furthermore as Figure 2 shows, there was not a great inter-individual variation either in the BC or healthy subjects when the basal DNA damage was considered. On the contrary, after irradiation a greater variation was observed in both groups: about 35% (12 of 34) controls and 40% (15 of 35) patients had values higher than the mean TD value (horizontal line). When RD values were considered, about 46–47% and 35% of both controls and patients showed higher RD values than mean RD value after 30 and 60 min, respectively.

Interestingly, we observed a great individual variation in mean DNA damage in both groups after irradiation but not at basal level, corresponding to the existence of an individual radiosensitivity, probably genetically determined and independent of cancer disease.

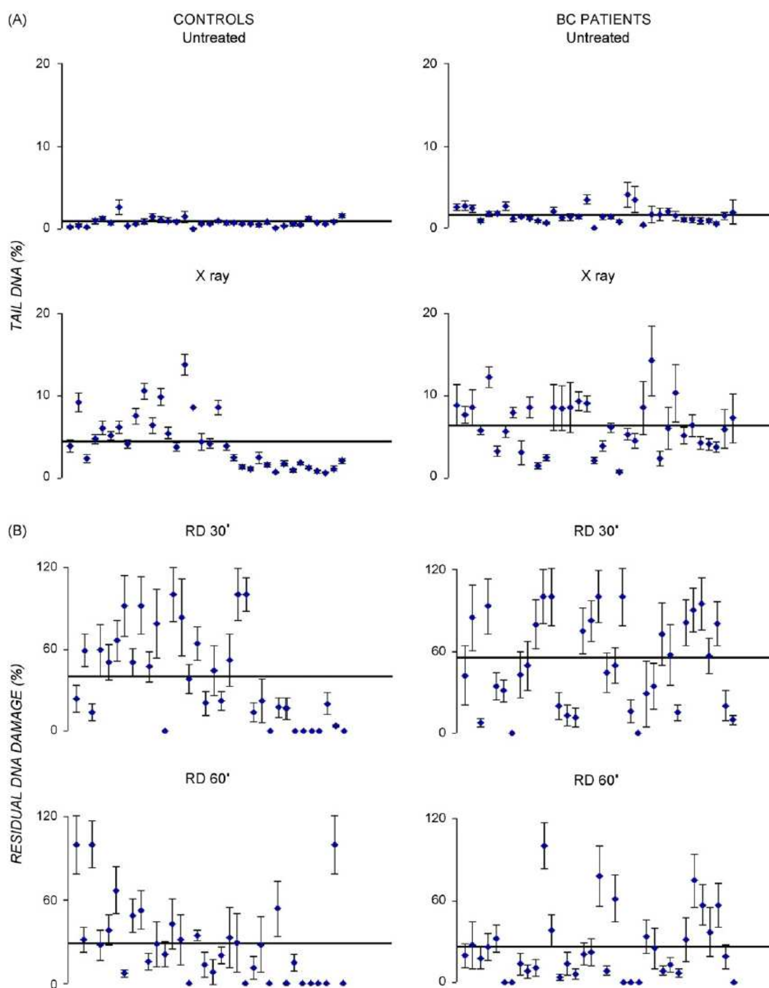

Fig.2 DNA damage in single BC patients and controls: A= basal and immediately after irradiation TD levels and B= residual damage (RD) 30 and 60 min later. Horizontal line represents the mean TD and RD value. (Taken from Sterpone *et al.*, 2010a)

In order to examine whether differences in the radiation response could influence the development of normal tissue reactions, we checked the relationship between the repair capacity and the acute side effects.
In Figure 3, the RD after 30 and 60 min from irradiation was reported in three groups of patients showing G0, G1 and G2–G3 adverse reactions following RTOG scale. In patients showing no adverse reactions (G0=15 patients) the RD at 60 min was significantly (p=0.0067) lower than at 30 min (50.2% vs 14.2%). On the contrary, in patients showing G1 (14 patients) and G2–G3 adverse reactions (6 patients) no significant reduction of residual damage was observed comparing the values at 60 and 30 min from irradiation (50% vs 29.4% and 63.4% vs 37.9%, respectively). The seeming increase in 60 min RD values among the groups did not show any significance at ANOVA test (p=0.21).

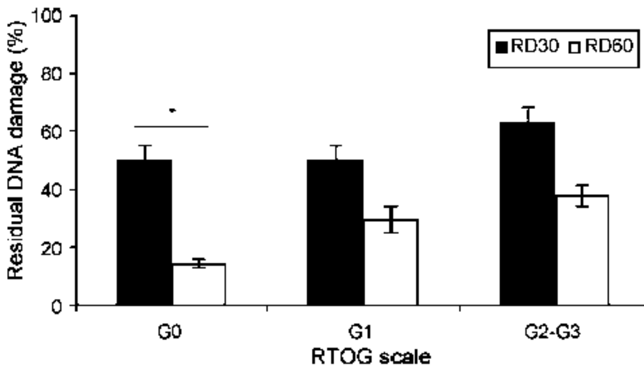

Fig.3 Residual DNA damage (RD) 30 and 60 min after irradiation in BC patients classified on the basis of RTOG scale. *p=0.0067 at Mann–Whitney U-test. (Taken from Sterpone *et al.*, 2010a)

In conclusion, in patients showing acute skin reactions (20 patients from G1 to G3 grade) DNA damage did not significantly decrease from 30 to 60 min of repair times. This result was particularly evident in 6 over-reactors who showed G2 and G3 reactions.
In these BC and control populations we examined the genotypes of the polymorphic variants of three DNA repair genes namely *XRCC1*, *OGG1* and *XRCC3*.
Firstly, the 34 healthy controls and an amount of 43 BC patients (the 35 BC patients not yet submitted to radiotherapy plus 8 patients who have already

undergone breast-conserving surgery) were genotyped for 3 SNPs located respectively in exon 10 of *XRCC1* (Arg399Gln), in exon 6 of *OGG1* (Ser326Cys) and in exon 8 of *XRCC3* (Thr241Met).
The genotype distributions and the variant allele frequencies are summarized in Table 2. The genotype distributions were in Hardy–Weinberg equilibrium for all SNPs in both the controls and BC patients. In the control population the *XRCC1* genotype distribution was close to H-W equilibrium being the p=0.06 at χ^2 test.

Table 2 The allele and genotype frequency and OR of the polymorphic variants of the *XRCC1*, *OGG1* and *XRCC3* genes in breast cancer patients and controls

Genotype or allele	Breast cancer patients (n = 43)		Controls (n = 34)		OR (95% CI)
	Number	Frequency	Number	Frequency	
XRCC1[a] gene (Arg399Gln)					
Arg/Arg	8	0.19	18	0.53	1 (reference)
Arg/Gln	24	0.56	10	0.29	5.4 (1.8–16.4)
Gln/Gln	11	0.25	6	0.18	4.1 (1.1–15.1)
Arg/Gln + Gln/Gln	35	0.81	16	0.47	4.9 (1.8–13.7)
Arg	40	0.46	46	0.68	1 (reference)
Gln	46	0.54	22	0.32	2.4 (1.2–4.7)
OGG1[a] gene (Ser326Cys)					
Ser/Ser	18	0.42	17	0.50	1 (reference)
Ser/Cys	23	0.54	15	0.44	1.5 (0.6–3.7)
Cys/Cys	2	0.04	2	0.06	0.9 (0.1–7.5)
Ser/Cys + Cys/Cys	25	0.58	17	0.50	1.4 (0.6–3.4)
Ser	59	0.69	49	0.72	1 (reference)
Cys	27	0.31	19	0.28	1.2 (0.6–2.4)
XRCC3[a] gene (Thr241Met)					
Thr/Thr	18	0.42	15	0.44	1 (reference)
Thr/Met	21	0.49	15	0.44	1.2 (0.5–3.0)
Met/Met	4	0.09	4	0.12	0.8 (1.8–3.9)
Thr/Met + Met/Met	25	0.58	19	0.56	1.1 (0.4–2.7)
Thr	57	0.66	45	0.66	1 (reference)
Met	29	0.34	23	0.34	0.9 (0.5–1.9)

[a] XRCC1 (Accession No. M36089); OGG1 (Accession No. NM 002542); XRCC3 (Accession No. AF035586).

(Modified from Sterpone *et al*., 2010a)

In the control population we found an allele frequency of 0.32, 0.28 and 0.34 for the variant alleles of *XRCC1*, *OGG1* and *XRCC3*, respectively, consistent with the literature data for Caucasian subjects (Tuimala *et al.*, 2002; Cornetta *et al.*, 2006).
On the other hand, in BC patients we observed a 0.54 frequency for *XRCC1-399* variant allele which was significantly different from *XRCC1* variant allele frequency in healthy controls [BC vs controls: OR=2.4 (95% CI 1.2–4.7) p=0.0095]. No difference in allele frequencies was found for the other SNPs in the other genes in BC patients when compared to control ones.

Moreover, a strong significant association between breast cancer occurrence and the presence of the *XRCC1-399* variant allele (Gln) was revealed: in particular p=0.0038 for heterozygous genotype (Arg/Gln) [BC vs controls: OR=5.4 (95% CI 1.78–16.43)] and p=0.0032 when both genotypes [homozygous variant allele genotype (Gln/Gln) + heterozygous] were considered [BC vs controls: OR=4.9 (95% CI 1.77–13.67)]. As far as homozygous variant allele genotypes, the p value was close to significance (p=0.058) for OR=4.1 (95% CI 1.13–15.10).
There was no significant association between the other genotypes and cancer occurrence.
At this point, we combined genotype data with DNA damage values obtained from the Comet assay and information concerning patients' acute side effects.
In order to check a possible association between the genotypes and the extent of basal and X-ray induced DNA damage, we applied the ANOVA Friedman test to compare the DNA damage in wild type vs heterozygous and homozygous genotypes for the considered genes in BC patients. No correlation was found between different genotypes and the amount of DNA damage measured by the Comet assay. In the same manner, when adverse effects were stratified for different genotypes, no significant associations were observed being the distribution of patients with wild type or variant genotypes quite similar in the three different groups of patients (G0 vs G1 vs G2–G3) [data showed in CD supplementary material section: Table I and Table II].

Secondly, to assess whether haplotypes in *XRCC1* and SNPs in multiple repair pathways might have a joint or additive effect on BC risk and on development of RT adverse reactions, we investigated two other SNPs in *XRCC1* gene: a variant located in 5'UTR (5'untranslated region), -77T→C, and a C→T substitution in codon 194 of exon 6 (Arg to Trp). The 43 BC patients and 31 controls were genotyped also for these two polymorphisms.
In Table 3, the genotype distributions and the association between these five studied SNPs (*XRCC1*: -77T/C; Arg194Trp; Arg399Gln; *OGG1* Ser326Cys and *XRCC3* Thr241Met) and BC risk are shown.
The genotype distributions were in Hardy–Weinberg equilibrium for all the SNPs in both the controls and BC patients ($p \geq 0.5$ at χ^2 test for each group).
In our study, the frequency of the variant T allele in the codon 194 of *XRCC1* in the controls was 0.05, according to previous literature data (Smith *et al.*, 2003); as far as position -77, in the controls the frequency of the variant C allele was 0.31. In the literature the frequency of the variant allele at this position has shown considerable variation: the C allele

frequency was 0.10/0.11 in Chinese subjects (Hao *et al.*, 2004; Hu *et al.*, 2005) and 0.40 in a French population (Brem *et al.*, 2006).
We found that the variant allele frequencies for the SNPs studied in *OGG1* and *XRCC3* were consistent with literature data for Caucasians (Tuimala *et al.,* 2002; Cornetta *et al.*, 2006).
The allele frequencies for all these genes found in BC patients were comparable to control ones and not associated with BC.
On the contrary, when we considered the *XRCC1-399* variant allele, BC patients showed a significant higher frequency (0.54) than healthy controls (0.32) and this SNP was significantly associated (p≤0.05) with breast cancer occurrence.

Table 3 The genotype distributions of the 5 SNPs and BC risk

SNPs	Cases (%) *N* = 43	Controls (%) *N* = 31	OR (95% CI)
XRCC1-399			
G/G	8 (18.6)	16 (51.6)	1.00 (Ref.)
G/A	24 (55.8)	10 (32.2)	**4.8 (1.56–14.78)***
A/A	11 (25.6)	5 (16.2)	**4.4 (1.13–17.08)****
XRCC1-194			
C/C	36 (83.7)	28 (90.3)	1.00 (Ref.)
C/T	7 (16.3)	3 (9.7)	1.82 (0.43–7.66)
T/T	0 (0)	0 (0)	–
XRCC1-77			
T/T	20 (46.5)	15 (48.3)	1.00 (Ref.)
T/C	19 (44.2)	13 (42)	1.10 (0.41–2.9)
C/C	4 (9.3)	3 (9.7)	1 (0.19–5.16)
OGG1-326			
C/C	18 (41.8)	15 (48.4)	1.00 (Ref.)
C/G	23 (53.5)	14 (45.2)	1.37 (0.53–3.56)
G/G	2 (4.7)	2 (6.4)	0.83 (0.10–6.65)
XRCC3-241			
C/C	18 (41.8)	14 (45.2)	1.00 (Ref.)
C/T	21 (48.8)	14 (45.2)	1.17 (0.44–3.09)
T/T	4 (9.4)	3 (9.6)	1.04 (0.20–5.41)

* Fisher's test $p = 0.007$, ** Fisher's test $p = 0.005$

(Modified from Sterpone *et al.*, 2010b)

In fact, when compared to subjects homozygous for *XRCC1-399* wild type allele (that we used as the reference group), both the heterozygous and the homozygous genotypes for variant allele showed an increased risk of developing breast cancer [p=0.007, OR=4.8 (95% CI 1.56-14.78); p=0.005, OR=4.4 (95% CI 1.13–17.1)], thus proposing that 399-Gln may act as a

dominant allele [Arg/Arg vs (Gln/Gln + Arg/Gln), OR=4.67 (95% CI 1.65–13.23), p=0.005 at Fisher's test].
In terms of *XRCC1* haplotypes, we combined the genotyping data for the SNPs at positions -77, and codons 194 and 399 in BC and control populations to derive seven possible haplotypes, which are summarized in Table 4.
In the controls the most frequently found haplotype (H1) contained the C variant at the position -77 together with the wild-type alleles at codons 194 and 399 (35.5%) and we considered this to be the reference haplotype. Interestingly it was present in only 11.6% of the cases.

Table 4 Association between haplotypes of *XRCC1* and BC risk

	Haplotypes[a]	Cases (%)	Controls (%)	OR (95% CI)
H1	C–C–G	5 (11.6)	11 (35.5)	1.00 (Ref.)
H2	T–C–A	15 (34.9)	9 (29)	3.67 (0.96–14)
H3	C–C–A	16 (37.3)	5 (16.1)	**7.04 (1.63–30)[b]**
H4	T–C–G	0 (0)	3 (9.7)	0.30 (0.01–6.86)
H5	T–T–G	1 (2.3)	2 (6.5)	1.1 (0.08–15.16)
H6	T–T–A	4 (9.3)	1 (3.2)	8.8 (0.77–100)
H7	C–T–G	2 (4.6)	0 (0)	10.45 (0.42–257)

[a] The haplotype is defined as the allele present at the position −77 (T→C); 194 (C→T); 399 (G→A), respectively

[b] Fisher's test $p = 0.009$

(Modified from Sterpone *et al.*, 2010b)

The haplotype (H4) based on the wild type alleles at the three positions was present in the controls (3/31 = 9.7%) but no in BC population.
In terms of BC risk, the haplotype H3 containing the wild type allele at codon 194 and the variant alleles at codon 399 and at -77 was significantly associated with increased BC risk [p=0.009, OR=7.04 (95% CI 1.63–30)]. This haplotype was found in the 37.3% of the cases and in the 16.1% of the controls.
In Figure 4, we show the distribution of the number of variant alleles for the SNPs analysed in the controls and breast cancer patients. Both populations presented a Gaussian distribution, as verified with the method of

Kolmogorov–Smirnov, and the most numerous group, both in the controls and in BC patients, was that with three variant alleles (12/31 and 18/43, respectively). Among the controls, about 45% (14/31) possessed less than three variant alleles and 55% was distributed among the groups with three and four variant alleles. In the BC population, 10 cases (23%) were found to possess one or two variants and the 77% showed three or more variant alleles. In particular we detected five BC subjects with five or six variant alleles and this situation was completely absent in the controls. Based on the statistical analysis conducted using the Fisher's test, the risk of breast cancer was significantly higher for subjects with ≥3 variant alleles compared to those with <3 variants [OR= 2.72 (95% CI 0.99–7.39), p=0.04].

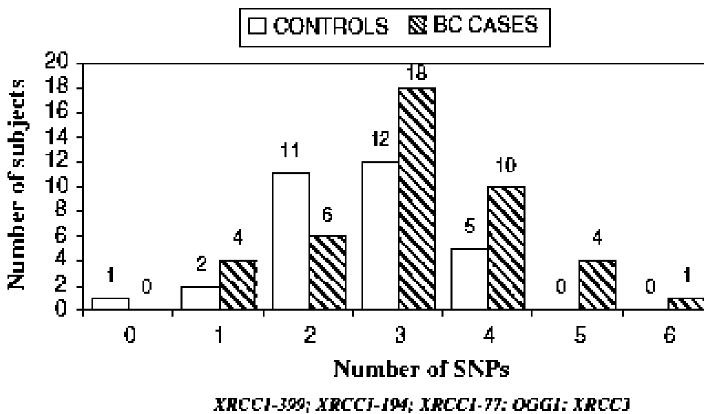

Fig.4 Distribution of the number of variant alleles of the SNPs *XRCC1* 399, 194, -77; *OGG1*; *XRCC3*, in the controls and BC patients. (Taken from Sterpone *et al.*, 2010b)

Additionally, we assessed a possible association between the number of variant alleles being carried and the risk of developing adverse side effects (Figure 5); among the BC patients without severe toxicity (G< 2), we found nine cases (21%) who had got a total of <3 variant alleles and 26 with three or more variants.

In contrast, among the eight patients who developed acute side effects (G≥ 2), 12.5% (1/8) had <3 variant alleles and the remaining ones possessed three or four variants as shown in Figure 5. The probability for the

development of Grade ≥2 toxicity seemed to be higher [OR=2.42 (95% CI 0.26–22.5)] for patients with ≥3 variant alleles compared to those with <3 variants, but p value was not significant (p=0.39) as can be seen by the large 95% CI.

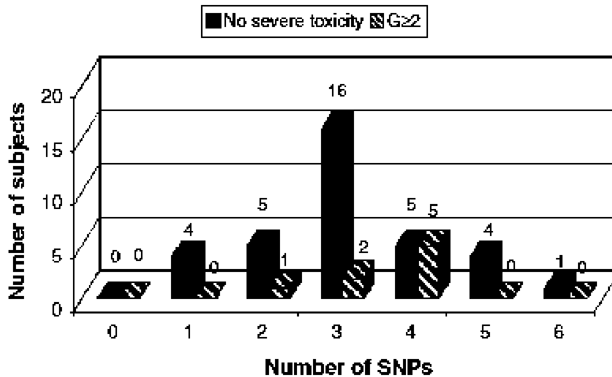

Fig.5 Distribution of the number of variant alleles of SNPs *XRCC1* 399, 194, –77; *OGG1*; *XRCC3* according to radiation-induced early side effects (Grade ≥2 vs Grade < 2). (Taken from Sterpone *et al.*, 2010b)

Two SNPs in the *RAD51* gene: a variant in exon 1 of 5'UTR, *RAD51-01* and a substitution in exon 10 within 3'UTR, known as *RAD51-52* were next genotyped in the 43 Italian BC patients and a cohort of BC patients enrolled in a French Oncology unit and their aged matched population based controls.

In Table 5, the genotype distributions and the variant allele frequencies of both these *RAD51* SNPs are summarised. The genotype distributions were in Hardy–Weinberg equilibrium for the SNPs in both the controls and BC patients.

For *RAD51-01*, the variant allele was found at a very low frequency in both populations and was in agreement with previous literature data (Antoniou *et al.*, 2007). On the other hand, the variant allele of *RAD51-52* was relatively common: 0.52 and 0.50 in the controls and BC patients, respectively. However, no significant differences in the frequencies of either SNPs was found between the breast cancer cases and controls, although a hint of an association was seen between the variant allele of *RAD51-01* and an increased risk of BC occurrence [OR=2.21 (95% CI 0.89-5.47)].

Table 5 The genotype distributions and the allele frequencies for SNPs in *RAD51* in BC patients and controls

	CONTROLS		BC PATIENTS		
RAD51-01 G>C	All 241		All 238		
	number	variant allele frequency	number	variant allele frequency	OR (95% CI)
GG	234	0.02	225	0.03	2.21 (0.89-5.47)
GC	7		11		*p = 0.06**
CC	0		2		
RAD51-52 T>C	All 324		All 293		
	number	variant allele frequency	number	variant allele frequency	OR (95% CI)
TT	82	0.52	77	0.50	1.10 (0.86-1.35)
TC	144		136		*p = 0.49**
CC	98		80		

* Fisher's test

(Taken from poster data presented at FISV Congress 2009)

In order to investigate if a possible association existed between these polymorphisms and the increased risk of developing side effects after radiotherapy, we stratified the information on early and late adverse reactions (classified according to the RTOG scale and the late side effects available only for French BC patients) for the different genotypes.
We found that the two *RAD51* SNPs did not show any significant association with either early or late adverse reactions (data showed in CD supplementary material section: Fig.1s and Fig.2s).

2. RAD51 expression and IR exposure

In four lymphoblastoid cell lines (LCLs), established from French BC patients, two of which are homozygous for the T wild type (BC108; BC26) and two for the C variant (BC98; BC231) allele of the *RAD51-52* SNP, we assessed whether IR treatment (5Gy of γ-rays) modulated the levels of *RAD51* mRNA and miR34a* and whether this was influenced by the genotype of the *RAD51-52* polymorphism.
In Figure 6 reported as RQ (Relative Quantitation), the basal levels of *RAD51* mRNA, *p21* mRNA and miR34a* are displayed. The induction of *p21* mRNA was used as a positive control for p53 activation that is found 2-4h after exposure to this dose of IR.

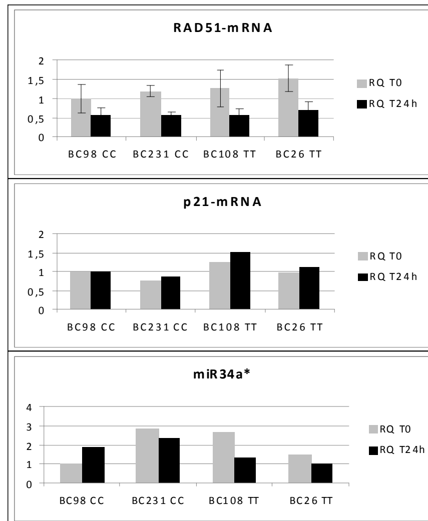

Fig.6 Basal levels of *RAD51* and *p21* mRNA and miR34a*. Constitutive gene expression was monitored at T0 and 24h later in untreated LCLs. For RAD51 we show the mean of three experiments while for p21 and miR34a* we show results obtained from one experiment by way of example. BC 98 line was used as the reference one. The genotype for the *RAD51-52* SNP is given for each cell line. (Taken from poster data presented at FISV Congress 2009)

In order to examine the constitutive levels of expression of *RAD51* and *p21* mRNA and microRNA 34a* the four cell lines were plated at the same cell density and left to grow overnight when a first RNA extract was prepared. The cultures were allowed to continue to grow for a further 24h when a second extraction was carried out. In all the four lines examined *RAD51* and *p21* mRNA and microRNA 34a* were expressed constitutively in a manner that appeared to be independent of the *RAD51-52* genotype. Interestingly the variation between the cell lines was less pronounced at 24h. This might reflect the saturation of the cell cultures and a reduction in the proportion of the cells in S-phase of the cell cycle. In order to investigate whether exposure to IR could modify the levels of *RAD51*, *p21* and miR34a* expression, we performed RT-qPCR analysis on RNA isolated at different time points after irradiation (2-4-8-24h). The RQ values are shown in Figure 7. Ionising radiation induced a small increase in the levels of *RAD51* mRNA, at early time (2h) that appeared to be independent of the *RAD51-52*

genotype; however by 4h and 8h post-irradiation a decrease in *RAD51* mRNA expression was noted.
As expected *p21* mRNA was induced by IR in the LCLs, in what is known to be a p53-dependent manner, and at 4h post-treatment expression levels were maximal in three out of the four LCLs.
miR34a* was induced by the treatment with IR but the four cell lines studied showed differences in the profiles, but this was independent of the *RAD51-52* genotype. It was noted that in at least three of the BC lines miR34a* reached a maximum 4h after exposure to IR and this induction time course paralleled that of p53 activation.

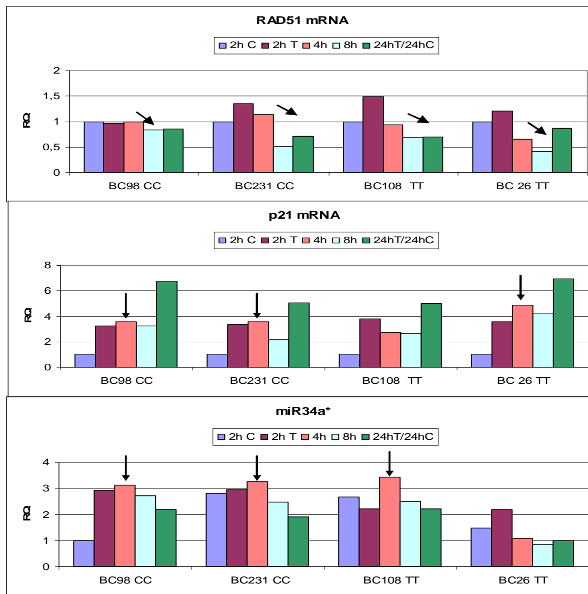

Fig.7 *RAD51* and *p21* mRNA and miR34a* levels induced by irradiation. Total RNA and miR were isolated from treated LCLs at 2-4-8-24h and at 2h and 24h also from untreated cell lines. (Taken from poster data presented at FISV Congress 2009)

In the same four LCLs, we also analysed the constitutive levels and levels after IR exposure of the RAD51 protein, together with that of p53 by western blotting. The basal RAD51 protein levels were different in the 4 LCLs but no association was observed with *RAD51-52* genotype, whilst that of p53 protein levels were similar in the four examined cell lines (Figure 8).

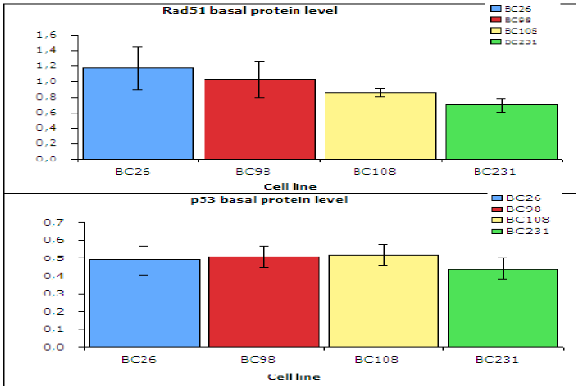

Fig.8 Constitutive levels of RAD51 and p53 protein. BC98 and BC231 lines were homozygote for the C allele whereas BC26 and BC108 for the T one. (Taken from poster data presented at FISV Congress 2009)

Following exposure to IR, p53 levels increased and with a dose of 5Gy its expression was at a maximum 4h after treatment; on the other hand, no marked differences in RAD51 protein levels were seen over this time period, as showed in Figure 9.

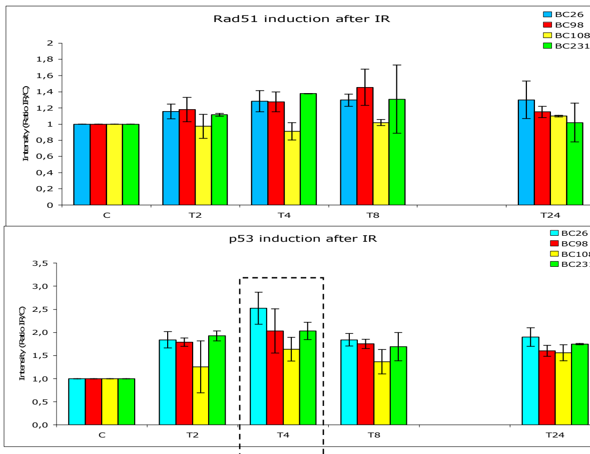

Fig.9 RAD51 (top) and p53 (bottom) protein levels after IR exposure. (Taken from poster data presented at FISV Congress 2009)

3. XRCC1 expression and IR exposure

The biological responses to IR exposure were also examined in two hamster cell lines, AA8 and EM9, which are a model to study the functional role of XRCC1.

First, in asynchronous cells we monitored *XRCC1* mRNA levels immediately after treatment with three different doses of X-rays: 1.25, 2.5 and 5Gy in order to check the direct effect of irradiation on mRNA molecules and the influence of a *XRCC1* gene mutation on its expression (EM9 cells).

The results of the quantitative RT-PCR performed on *XRCC1* transcripts from the two CHO cell lines are shown in Figure 10. The fold-induction of *XRCC1* gene expression with respect to the basal level after treatment was expressed as 2^(-ΔΔCt) [2^(-DDCt)].

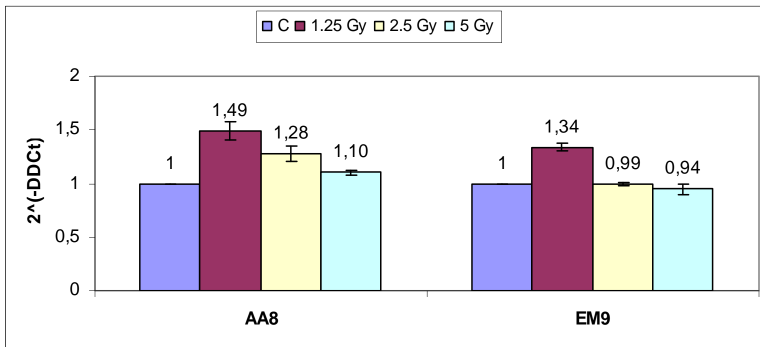

Fig.10 Fold-induction of *XRCC1* mRNA levels in AA8 and EM9 after exposure to different doses of X-rays. The graph represents the mean of mRNA activation obtained from three different experiments with their respective SE.

The two cell lines seemed to respond similarly to treatments. Although irradiation with 1.25Gy determined a small increase of *XRCC1* mRNA level in both cell lines, variation in *XRCC1* transcript levels was not significant at one-way ANOVA test ($p=0.15$ and $p=0.23$ for AA8 and EM9, respectively). However, AA8 and EM9 differed significantly in absolute levels of *XRCC1* mRNA (Figure 11); two-way ANOVA test confirmed that changes due to irradiation were not significant ($p=0.27$) and revealed that in EM9 *XRCC1* mRNA levels were significantly lower than in AA8 both before and after treatments ($p=0.001$).

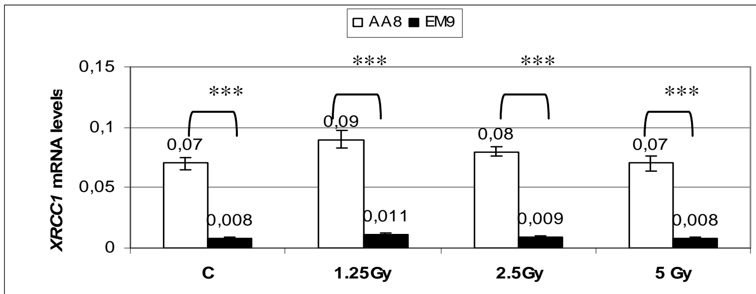

Fig.11 *XRCC1* mRNA levels in AA8 and EM9, before and after irradiation. The graph represents the mean of *XRCC1* mRNA levels, normalized with housekeeping β-actin, obtained from three different experiments with their respective SE. Two-way ANOVA: ***p=0.001

We also checked the effect of irradiation with three different doses at protein level by western blotting (Figure 12). In AA8, XRCC1 protein levels seemed to be unchanged immediately after 1.25 and 2.5Gy treatments when compared to control but a reduction could be noted with a dose of 5Gy. On the contrary, in EM9 XRCC1 protein was completely absent both in control and in irradiated cells, as expected.
β-actin levels remained constant in both cell lines, even after IR treatment.

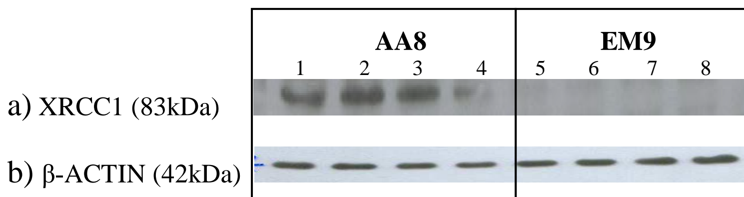

Fig.12 XRCC1 (a) and β-ACTIN (b) protein levels, detected by western blotting in AA8 (lane 1- 4) and EM9 (lane 5-8) before (lane 1 and 5) and immediately after IR with 1.25Gy (lane 2 and 6), 2.5Gy (lane 3 and 7) and 5Gy (lane 4 and 8).

Afterwards this analysis in asynchronous cells, we next investigated *XRCC1* expression in the different phases of cell cycle both at the basal level and immediately after irradiation with 2Gy. Cells were synchronized by adding

1% DMSO to complete medium for 96h and analysed through cytofluorimetric analysis (FCA) of DNA content and of BrdU incorporation. (FCA plots showed in CD supplementary material section: Fig.3s, Fig.4s and Table III).

XRCC1 mRNA levels were expressed as β-actin normalized values with reference to G_0 control condition. Figure 13 and 14 display *XRCC1* mRNA relative levels observed in AA8 and EM9 cell lines, respectively.

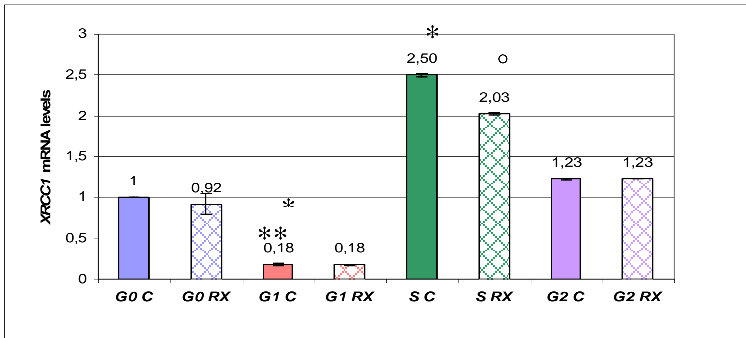

Fig.13 *XRCC1* mRNA relative levels in different phases of AA8 cell cycle. Samples irradiated with 2Gy of X-rays are shown as grilled bars. The graph represents the mean of values obtained from three different experiments with their respective SE. Student's *t*-test: *p<0.05; **p<0.01. Student's *t*-test when comparing untreated and irradiated sample: °p<0.05

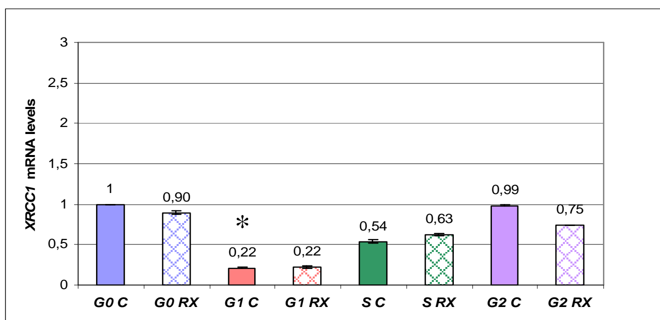

Fig.14 *XRCC1* mRNA relative levels in different phases of EM9 cell cycle. Samples irradiated with 2Gy of X-rays are shown as grilled bars. The graph represents the mean of values obtained from three different experiments with their respective SE. Student's *t*-test: *p<0.05

In both cell lines, *XRCC1* mRNA basal levels differed significantly through the cell cycle as confirmed by one-way ANOVA test (AA8 $p<0.001$; EM9 $p=0.003$). In untreated AA8 cells, we observed significantly higher levels of *XRCC1* transcripts in S phase than G_0 ($p=0.04$) and G_1 ($p=0.04$) and significantly reduced levels in G_1 phase when compared to S ($p=0.04$) and G_2 ($p=0.01$) phases. Also the EM9 cells showed a significant decrease of *XRCC1* mRNA levels in G_1 as regards G_0 ($p=0.02$). On the contrary, EM9 did not exhibit an increase of *XRCC1* mRNA during the replicative phase instead a decrease in levels was observed when compared to RNA from cells in G_0, even though this difference did not reach statistical significance ($p=0.16$).
In conclusion the IR exposure did not influence *XRCC1* mRNA levels in the different cell cycle phases either in the AA8 or in the EM9 cells, except for a significant decrease in levels from 2.5 to 2.03 in S phase in AA8 cells ($p=0.03$).
To further study the *XRCC1* expression profile in response to irradiation, we assessed *XRCC1* mRNA expression time course after exposure to 2Gy of X-rays to quiescent (G_0) peripheral blood mononuclear cells isolated from 5 male healthy subjects.
Figure 15 resumes the results of the quantitative RT-PCR. The fold-induction of *XRCC1* gene expression with respect to the basal level after irradiation and recovery at different time points was expressed as 2^(-DDCt).
The upper part of Fig.15 displays the individual profiles of *XRCC1* gene expression obtained from each of five PBMCs cultures whereas the mean values and their respective standard error (SE) are shown at the bottom of the figure.
As the graphs show, the five profiles did not exhibit a great inter-subject variability and the radio-induced *XRCC1* mRNA levels changed significantly in a time-dependent manner as confirmed by one-way ANOVA test ($p<0.0001$).
In contrast, in untreated cells *XRCC1* expression levels remained constant for the two hours following the cell culture establishment (red line at the bottom of Fig.15), thus confirming that *XRCC1* transcript levels were indeed modified specifically by the IR treatment.
In detail, after X-ray treatment a significant increase of *XRCC1* mRNA levels were noted 90 min after treatment in comparison to the levels seen in control cells (3.87 vs 1, $p=0.0006$) and 0 min (3.87 vs 0.84, $p=0.0003$) levels. Indeed, already at 60 min after irradiation a significant, but less pronounced, enhancement in *XRCC1* mRNA expression was noted

compared to the controls (1.46 vs 1, p=0.002) and 0 min (1.46 vs 0.84, p=0.02) levels.
In addition to extracting RNA from isolated PBMCs we studied the repair kinetics of radio-induced DNA damage using the Comet assay taking as recovery times: 0, 15, 30, 60, 90 and 120 min.
The basal level (untreated) and the X-ray induced DNA damage measured as % of DNA in the Tail (TD) are shown in Figure 16.

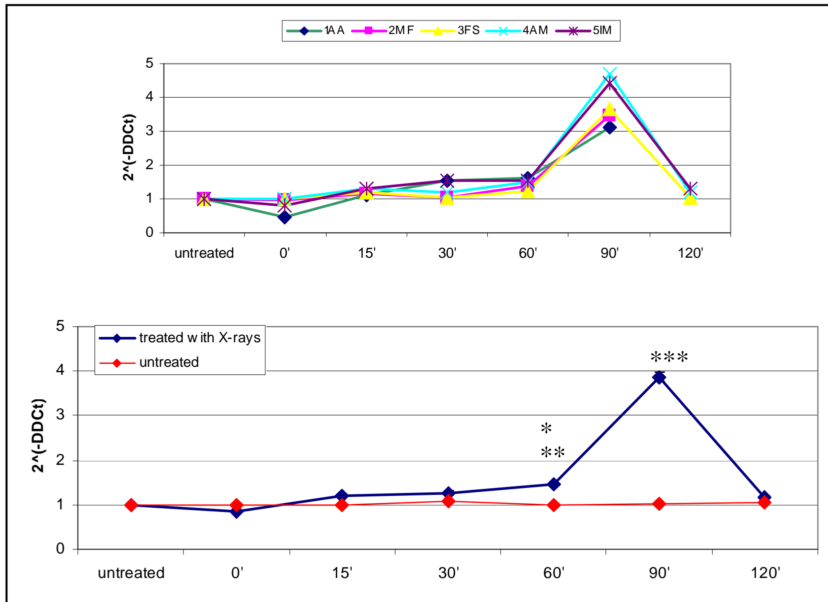

Fig.15 Fold-induction of *XRCC1* mRNA after treatment with 2Gy of X-rays. At the top individual kinetics profiles are shown; the bottom reports the mean of data obtained from untreated (red) and treated (blue) cells and their respective SE Student's *t*-test: *p<0.05, **p<0.01, ***p< 0.001

At the top of Fig.16, G_0 kinetics profiles for each of the five samples are shown separately whereas at the bottom the TD mean values are displayed. The TD is significantly modified in a time-dependent manner as shown by one-way ANOVA test (p<0.0001).
2Gy of X-ray treatment resulted in an immediate (0') 2.2 fold-increase in DNA damage compared to the basal DNA damage level (3.34 vs 1.55, p= 0.001). However, from 15 min after irradiation a gradual and significant

decrease in damage levels were measured (3.34 vs 2.43, p=0.04; 3.34 vs 1.65, p=0.04; 3.34 vs 1.17, p=0.001; 3.34 vs 0.84, p=0.0007; 3.34 vs 0.6, p<0.0002); this trend indicated that DNA repair had occurred.
By 30' and 60' after IR exposure, DNA damage levels reached basal level (1.55 vs 1.65, p=0.65; 1.55 vs 1.17, p=0.15) so suggesting that DNA repair mechanisms, mainly global rapid SSBR and NHEJ, operated quickly to repair radio-induced damage.

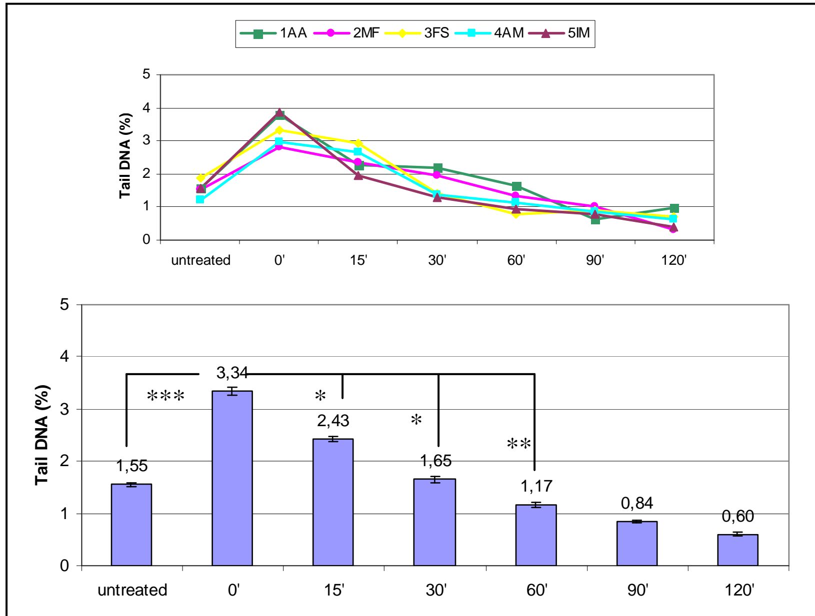

Fig.16 Basal and X-ray induced DNA damage expressed as Tail DNA. The Comet assay was performed in untreated condition (0 Gy), immediately after irradiation with 2Gy (0') and 15, 30, 60, 90, 120 min later. Individual and mean TD values with their SE are shown at the top and at the bottom, respectively. Student's *t*-test: *p<0.05, **p<0.01, ***p< 0.001

By 90' and 120' post-treatment TD value did not change significantly (0.84 vs 0.6, p=0.22) but the values were lower than the basal levels measured. This phenomena is probably due to treatment which, stimulating DNA repair pathways, could also promote the repair of endogenous DNA damage, that is revealed by the Comet assay.

IV. DISCUSSION and CONCLUSION

The DNA damage repair capacity is considered a genetic trait which can influence the individual susceptibility of normal human subjects to chemical and physical exposure. Each subject differently reacts to external stimuli, activating a series of biological responses including DNA repair pathways. The alkaline Comet assay is one of the most commonly used tests to analyse both the induction and the repair kinetics of DNA damage. It is also useful for evaluating the baseline level of DNA damage in human subjects not exposed to damaging agents (Collins, 2004). Reduced DNA repair capacity could be the underlie cause of abnormal responses in patients therapeutically exposed to agents damaging biological macromolecules such as is seen in some cancer patients treated with standard radiotherapy regimes. In this field it seems particularly important to identify simple and rapid methods as predictors of side effects**.**
During my PhD program, firstly I evaluated the presence of both basal and X-ray induced DNA strand breaks in BC patients and in control population. The data obtained showing that BC patients had a higher extent of basal DNA damage are in agreement with other recently published data (Smith *et al.*, 2003; Shahidi *et al.*, 2007; Sánchez-Suárez *et al.*, 2008; Synowiec *et al.*, 2008); furthermore our results show a higher mean value of induced damage immediately after irradiation in BC patients compared to healthy controls. Both basal and radio-induced DNA damage levels were independent of patients' genotypes with respect to the *XRCC1-399*, *OGG1-326* and *XRCC3-241* polymorphisms.
As far as repair capacity, in both groups (patients and controls) DNA damage persisted at a significantly high level even 60 min after irradiation. When considering the RD, which takes into account the initial TD value obtained immediately after irradiation, we found that a comparable amount of un-rejoined DNA breaks was still detectable both in BC patients and in the controls. This result is not in agreement with Shahidi *et al.* (2007) who showed that after a much longer repair time (24 h) the controls did not have any residual damage whereas BC patients had more than 20%. They used, however, a standardized residual damage that did not consider the initial TD value immediately after irradiation.
Interestingly, we observed a great inter-individual variability in mean DNA damage in both groups after irradiation but not at basal levels, corresponding to the existence of an individual radiosensitivity, probably genetically determined and independent of cancer disease.

In order to examine whether differences in the radiation response could influence the development of normal tissue reactions, we checked the relationship between the repair capacity and the adverse side effects. An interesting outcome was obtained showing a relation between higher degrees of adverse reactions (as evaluated on the basis of ROTG scale) and the residual DNA damage: in patients showing acute skin reactions (20 patients from G1 to G3 grade) DNA damage did not significantly decrease from 30 to 60 min of repair times. This result is particularly evident in 6 over-reactors who showed G2 and G3 reactions. A similar result was reported by Alapetite *et al.* (1999) in 3 over-reacting BC patients but not by Popanda *et al.* (2003) who failed to find any significant association between the clinical signs of radiation sensitivity and DNA repair parameters measured *in vitro*. It will be interesting to follow our over-reacting patients (particularly those showing G2 and G3 grades) for late clinical symptoms which can develop several months after therapy (Bentzen, 2006).

When we calculated the Odd Ratios for the DNA damage gene polymorphisms, we found a significant association between the *XRCC1-399* variant allele and the risk of developing sporadic breast cancer: the 399-Gln allele of *XRCC1* increased significantly the risk of sporadic BC and it may act as a dominant allele.

In the literature, however, studies on association between this *XRCC1* polymorphism and breast cancer have yielded conflicting results. Recently, Huang *et al.* (2009) performed a meta-analysis to investigate this association in 37 studies (22,481 cases and 23,905 controls) in different inheritance models. Our data are supported by several studies that showed a slightly increase of BC risk in Caucasians using dominant model (Duell *et al.,* 2001; Moullan *et al.,* 2003; Smith *et al.*, 2003; Zhang *et al.,* 2006), however they are partially in contrast with other works such as Saadat *et al.* (2007) who observed that 399-Gln allele may act as a recessive allele and increase the BC risk, Costa *et al.* (2007) who reported that women carriers of *XRCC1* 399-Gln genotypes had a protective effect concerning breast cancer and Breast Cancer Association Consortium (2006) which found no evidence of an association of breast cancer with *XRCC1* Arg399Gln polymorphism. In conclusion, based on our results and supported by the meta-analysis study (Huang *et al.,* 2009), individuals who have the Gln allele in codon 399 are more likely to develop breast cancer.

Additionally, *XRCC1* haplotype analysis revealed that the 399-Gln variant allele when combined with the wild type allele at codon 194 and the variant allele at the position -77 (haplotype H3) determined a significant higher BC risk.

To date, only one study has described the analysis of haplotypes considering the SNP in the *XRCC1* promoter in BC patients and healthy controls (Brem *et al.*, 2006). Our data are partially in agreement with Brem *et al.*'s work, where *XRCC1* haplotypes based on alleles at position -77 and in codons 194, 280 and 399 were determined in French breast cancer patients and controls. In fact, also in their study the most frequent haplotype contained the C variant at position -77 together with the wild-type alleles at the remaining *loci*, both in the controls and BC population. They also reported a quite similar frequency for the haplotype with wild-type alleles in all four positions (11.8% of controls vs 9.7% found in our control population); however, the H3 haplotype, significantly associated with BC risk in our analysis, was not represented in their study. In terms of BC risk, they concluded that risk was increased in the haplotype containing the wild-type alleles at -77, codon 194 and 399 and the variant allele at codon 280, even though the p value was not significant.
XRCC1 has two BRCA1 carboxyl-terminal (BRCT) domains (BRCT I and BRCT II), located centrally and at the C-terminal end, respectively. BRCT II is responsible for binding and stabilizing DNA ligase III and the centre of BRCT I regulates recognition of protein PARP1. This last one domain is critical for efficient SSBs repair and cell survival (Taylor *et al.,* 2002). The polymorphism Arg399Gln is located close to BRCT I's C-terminal boundary; this mutation will change XRCC1's structure, in particular at level of secondary structures as Monaco *et al.* (2007) demonstrated and so it could impair DNA repair. On the other hand, promoter T/C substitution might disrupt a consensus sequence for Sp1-binding site (Hao *et al.*, 2006), so that this polymorphism may have the potential to alter *XRCC1* transcription. On the basis of these considerations, the combination of variant alleles in position –77 and in codon 399, as represented by the haplotype H3, could affect strand breaks repair as a consequence of the reduced availability of *XRCC1* transcript, even in the variant form. In this manner, we could explain the increased risk in developing cancer among subjects with H3 haplotype. In conclusion while this study has several limitations, such as the moderate sample size of cases and controls, the haplotype risk associations can be considered preliminary and suggest that association studies in which only individual *XRCC1* SNPs are considered, might lead to errors in risk estimation. Moreover, it would be interesting to perform molecular dynamic studies to value functional alterations in XRCC1 protein encoded by gene with variant allele in promoter and codon 399.
XRCC1-77, *XRCC1-194*, *OGG1-326*, *XRCC3-241*, *RAD51-01* and *RAD51-52* as individual SNPs were not associated with BC development.

In terms of the association of SNPs in DNA repair genes (*XRCC1*; *OGG1*; *XRCC3* and *RAD51*) with the occurrence of radiotherapy-induced side effects, we did not find any significant associations. Data in the literature are not conclusive since there are two studies where *XRCC1*-399Gln was found associated with an increased risk of side effects (Andreassen *et al.*, 2003; Sánchez-Suárez *et al.*, 2008), one reporting a protective effect exerted by the same variant (Chang-Claude *et al.*, 2005) and two more with no effect (Moullan *et al.*, 2003; Andreassen *et al.,* 2005). Less is known about the role of other repair gene polymorphisms and radiation treated breast cancer patients (Popanda *et al.,* 2009). However, there are evidences that *XRCC1* gene variants could contribute to the risk of both acute and late side effects also in combination with other gene variants (Azria *et al.*, 2008).
In our study we did not find a significant association between the number of variant alleles of SNPs located in *XRCC1*, *OGG1* and *XRCC3* genes, and the risk of developing acute adverse reactions after RT. Though clinical normal tissue radiosensitivity should be considered as a complex trait dependent on the combination of variants in several genes, the product of which play an important role in radiation response (Ho *et al.,* 2006), we failed to find an association. Furthermore, the development of side effects after radiotherapy is not only dependent on the type of SNP but also on the number of risk alleles, as Azria *et al.* (2008) demonstrated. They, assessing whether patients with several radiation-induced sequelae (G> 3) possessed certain SNPs located in six genes (*ATM, SOD2, XRCC1, XRCC3, TGFB1* and *RAD21*), concluded that patients who had 4 or more variant alleles in candidate genes showed a significant higher risk in developing severe toxicity. This result, which is in contrast to our data, could be due to the fact that in our cohort of breast cancer patients there were only eight cases who developed acute side effects (G>2) after radiotherapy, while in Azria's analysis, patients with different types of tumour were included, many of them (47%) exhibiting grade 3 toxicity.
In our study we demonstrated that the combination of variant alleles of SNPs in several genes (*XRCC1*, *OGG1* and *XRCC3*), involved in repair mechanisms, increases the risk of developing sporadic breast cancer. In fact, the risk of breast cancer was significantly higher for subjects with ≥3 variant alleles compared to those with <3 variants. This result may be comment on the fact that the genetic variants in multiple repair pathways may have a joint or additive effect on BC risk.
In the case of XRCC1, it itself does not have a catalytic activity in BER but since *XRCC1*-399Gln shows a reduced activity (Taylor *et al.,* 2002), it is possible that this SNP may affect the recruitment of the BER proteins. If BER, in which XRCC1 and OGG1 are involved, or HRR that requires

XRCC3, are erroneous the increased mutagenesis would be expected to modify breast cancer risk.
Based on the results obtained from this first part, we highlight *XRCC1* as a possible genetic marker to assess the risk of developing sporadic breast cancer. This consideration moved us on to study in detail XRCC1 function in BER and SSBR pathways, by using *in vitro* systems; we focused on studying the *XRCC1* expression in response to IR exposure.
Firstly, evaluating the immediate effects of X-rays on gene transcription we observed that after irradiation with several doses (1.25, 2.5 and 5Gy) the *XRCC1* mRNA levels did not significantly change either in AA8 or EM9 cell lines. On the contrary, levels of the XRCC1 protein, detected only in AA8 but not in XRCC1-defective cell line EM9 (Caldecott *et al.*, 1994; Shen *et al.*, 1998), seemed to be reduced after treatment with 5Gy.
Based on these observances, we can reasonably conclude that the average of X-ray dose generally employed as single fraction (2Gy) in radiotherapy regimen does not affect *XRCC1* messenger and protein levels, by causing a direct damage, but it has been noted that this is a hamster model.
For the first time to our knowledge we demonstrated that in the EM9 cells the C→T substitution at nucleotide 661 in *XRCC1* gene is responsible as well as for XRCC1 protein absence, for a significantly reduced gene expression; indeed, the EM9 cells showed about a ten-fold reduction of *XRCC1* transcript levels compared to AA8 cells, both before and after IR exposure. Our hypothesis is likely that the lack of functional protein regulates gene expression through a negative feedback mechanism, or that *XRCC1* transcript levels are reduced by nonsense-mediated mRNA decay in EM9 cells.
With regard to *XRCC1* expression in synchronized CHO-derived cells, we argue that *XRCC1* gene is expressed differentially through the cell cycle, also in absence of treatment. In particular in the positive control, AA8, *XRCC1* mRNA levels were maximum in S phase during which XRCC1 protein assists in DNA replication. In fact, as Lévy *et al.* (2009) demonstrated, XRCC1 directly interacts with the p58 subunit of DNA Polα-primase complex, which begins DNA replication by RNA primers synthesis. Moreover, the reported *XRCC1* induction in the replicative phase is supported by other studies that reveal the *XRCC1* promoter is directly bound by transcription factors commonly involved in the progression into the DNA replication phase (Tan *et al.,* 2007; Chen *et al.,* 2008).
To summarise, we consider that *XRCC1* is maximally expressed in the S phase so that the S/G_2 specific XRCC1-dependent SSBR described by Caldecott (2003), can operate properly.

IR exposure did not markedly influence *XRCC1* mRNA levels in the different cell cycle phases either in the AA8 or the EM9 cells, except for a significant decrease in S phase in AA8 cells. We hypothesise that this destabilizing effect of X-rays is linked to the active gene expression status of *XRCC1* in the replicative phase; in fact, the *XRCC1* RNA molecules localized in the cytoplasm waiting to be translated, probably are more susceptible to direct (DNA breaks) and indirect (oxidative stress) action of X-rays than in the other cell cycle phases.
As far as the response to IR of quiescent cultured peripheral blood mononuclear cells, we can conclude that the radio-induced DNA damage is quickly repaired mainly by the XRCC1-dependent global rapid single strand break repair (global rapid SSBR) pathway, which operates throughout the cell cycle and by which SSBs are rejoined within 0.5-3h (Caldecott, 2003). Moreover, SSBR is supported by NHEJ, the error-prone pathway that occurs to repair DSBs in G_0 cells and in which XRCC1 results to take part, too. (Audebert et al., 2004; Lévy et al., 2006)
Our results on repair kinetics, which indicate that the radio-induced DNA damage is repaired very quickly after exposure, are in agreement with Mosesso *et al.* (2010). In this paper the authors displayed a very fast rejoining of DNA breaks in irradiated (3Gy of X-rays) G_0 isolated lymphocytes: within 0.5 h recovery time, the Tail moment (TM) value, measured by the Comet assay, was reduced of 50% as regards TM at 0h. As well as Mayer *et al.* (2002) reported that in isolated lymphocytes seventy-five percent of DNA damage (measured as TM through the Comet assay) was repaired already 15 min after irradiation. It is noteworthy that in this case irradiation was performed with 5Gy of γ-rays.
We also show that X-ray exposure in G_0 mononuclear cells specifically caused a *XRCC1* induction in a time-dependent manner; 90 min post-treatment it was remarkable a significant increase of *XRCC1* transcripts in comparison to baseline level.
So far, conflicting results from a few studies into the damage inducibility of *XRCC1* mRNA following exposure to IR exist. Moreover, due to the heterogeneity in term of doses, exposure condition and methodologies used, it is difficult to reach a consensus with respect to the modulation of *XRCC1* expression.
In the 90's, methods of quantitative dot plot hybridization and northern blot techniques did not show any convincing evidence for *XRCC1* induction after ionizing radiation exposure, either in CHO or in human cell lines (Thomson et al., 1993). Afterwards, in 2006 Sudprasert and co-workers demonstrated a significant decrease in *XRCC1* and *OGG1* expression in isolated lymphocytes immediately after exposure to 20cGy of gamma rays.

They evaluated gene expression by quantifying bands of reverse transcribed mRNAs (cDNAs) migrated on agarose gel by densitometry (Sudprasert *et al.*, 2006). Even due to differences in experimental approaches, these results are, therefore, in conflict with ours that displayed no significant changes in *XRCC1* mRNA levels within 60 min post-irradiation.

Furthermore, literature data obtained by quantitative RT-PCR analyses suggest that the basal expression level of XRCC1 was most likely a determinator of radiation inducibility of this repair protein: cells with a high basal XRCC1 expression did not show radiation-induction whereas cells with low basal expression did. Indeed, two papers (Yacoub *et al.* 2001; Toulany *et al.*, 2008) demonstrated an up-regulation of XRCC1 mRNA and protein after IR treatment in human tumour cell lines (DU145 and MO59J).

However, whereas Yacoub and colleagues concluded that protein levels increased through *de novo* transcription of *XRCC1*, Toulany's results implied a stabilization of XRCC1 protein by post-translation modification, i.e. phosporylation/dephosphorilation rather than new synthesis.

In G_0 peripheral blood mononuclear cells we plan the study of XRCC1 protein expression to test our hypothesis that in quiescent PBMCs IR exposure, at clinically relevant doses, causes firstly an increase of *XRCC1* mRNA levels and secondly an induction at protein level through *de novo* synthesis. This increase in XRCC1 protein would guarantee the repair of radio-induced DNA damage, that we have already checked using the Comet assay.

Over the last ten years expression profiling with oligonucleotide microarrays has opened up new possibilities for large-scale gene expression studies. In particular, it also seems to be a useful method for detecting subtle changes in transcription following exposure to ionising radiation (Amundson *et al.*, 2000; Amundson *et al.*, 2001). Many studies, as reviewed by Roy *et al.* in 2009, have been used microarray technique to understand cellular response to ionizing radiation by gene expression patterns analysis. However, to date there are not convincing results of an induction of *XRCC1* gene expression after irradiation.

Through expression analysis in LCLs, we demonstrated that IR treatment induced the microRNA34a* expression, as result of p53 activation. Our data are supported by works of Chang *et al.* (2007) and Raver-Shapira *et al.* (2007): they showed miR34a* as a direct transcriptional target of p53 and that its expression was increased by p53 in response to genotoxic stress and irradiation both *in vivo* and *in vitro* systems. Previous data have displayed that p53 is able to down-regulate RAD51 mRNA and protein levels, by a direct binding at a site in the promoter and a direct protein-protein interaction that prevents RAD51 polymerisation (Arias-Lopez *et al.* 2006).

As a consequence of p53 activation we expected a down-regulation of RAD51 mRNA and protein and this was confirmed experimentally with a decrease of *RAD51* transcript levels starting from 4h post-irradiation being observed.
However, contrary to theoretical bioinformatic analysis which suggested that the miR34a* binding would be different on the basis of *RAD51-52* genotype, our experimental results indicated that *RAD51* mRNA levels did not change depending on the allele present of *RAD51-52*. The miR34a* binding in the 3'UTR of *RAD51*, therefore, is not influenced by *RAD51-52* polymorphism, and it does not modify *RAD51* levels, under our experimental conditions.

On the whole, this PhD research highlights that it is an important goal of biological and clinical research to detect genetic components such as DNA repair gene polymorphisms as possible markers of radiosensitivity in order to adjust radiotherapy protocols for both sensitive and resistant patients and to promote the development of a "personalized medicine", in which therapies are optimised for each individual's unique genetic makeup.

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