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ARE RARE FRAGILE SITES SENSORS OF GLOBAL GENOME DAMAGE?

Bachelor Thesis

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Abstract

Fragile sites of the genome have long been known as a specific type of DNA lesions, which arises from an imperfect completion of replication before the cell enters mitosis. Rare fragile sites are present only in a small part of the human population. Thus, they seem to be generally less attractive than common fragile sites, which are an intrinsic part of the human genome. However, the molecular basis of rare fragile sites is examined better.

Being a DNA damage, fragile sites are subjects of the cellular mechanisms leading to their repair. Unrepaired lesions might evolve to more dangerous forms, which may contribute to genomic rearrangement typical of malignant transformation. It is therefore possible that their persistent expression might indicate the cellular environment prone to neoplastic development.

Keywords: fragile sites, genomic instability, perturbed replication, double strand breaks, cancer

Abstrakt

Fragilní místa genomu jsou známa jako zvláštní typ poškození DNA, které vzniká kvůli neúplnému dokončení replikace před vstupem buňky do mitózy. Vzácná fragilní místa se vyskytují pouze u malé části lidské populace. Obecně by tedy mohla být tedy méně atraktivní než běžná fragilní místa, jež jsou normální součástí lidského genomu, přesto je lépe prozkoumána molekulární struktura vzácných fragilních míst.

Fragilní místa -coby specifický druh poškození DNA- jsou předmětem buněčných opravných mechanismů. Pokud tyto léze nejsou opraveny, vznikají nebezpečnější formy poškození, které přispívají k přestavbám genomu typickým pro maligní transformaci. Je tedy možné, že přetrvávající exprese fragilních míst je jedním z mechanismů vedoucích k vývoji neoplastických lézí.

Klíčová slova: fragilní místa, nestabilita genomu, poruchy replikace, dvojřetězcové zlomy, rakovina

Contents

1. Introduction

Maintenance of the genome in its intact form is important for a proper function of the whole cell, which is necessary for the long-term survival of multicellular organisms. This stability may be disrupted as a consequence of various environmental factors (e.g. ionizing irradiation) or damaging products of normal metabolism as free oxygen radicals. Both exogenous and endogenous agents cause mutations or lesions on DNA, which may be harmless, but more often deleterious or even lethal. During the evolution, cells created several mechanisms to protect themselves from the consequences of these potentially dangerous events. There is a number of distinct repair mechanisms arising from the variety of DNA lesions. Besides the direct repair, the DNA damage response comprises cell cycle checkpoints, which halt cell cycle progression until the repair process is completed, and programmed cell death. When any component of this machinery is altered, neoplastic development is likely to occur (Kastan and Bartek, 2004).

Fragile sites are a specific type of DNA damage. They represent lesions unrepaired during cell cycle interphase. Their appearance on metaphase chromosomes indicates that some proportion of the aberrations escaped checkpoint controls (Casper et al., 2002).

2. Rare fragile sites

2.1. Definition and categorization of fragile sites

Fragile sites are specific chromosomal loci, visible as constrictions or gaps on metaphase chromosomes, which appear after a specific cell culture treatment (Figure 1) (Sutherland, 1979). They were first observed in blood cells of a woman, who had been previously repeatedly treated by x-ray irradiation for recurrent eczematous dermatitis (Debakan, 1965). Before one of them was linked to fragile X syndrome, fragile sites had been considered to be a cytogenetical artefact. Nowadays, many of their intriguing properties have been found out, including their involvement in genomic instability and tumorigenesis (Sutherland et al., 1998). The list of all published fragile sites is given in Table 1.

According to their frequency within the population, fragile sites were classified as common or rare. Common (or constitutive) fragile sites have been found in genomes of all individuals (Arlt et al, 2003), while rare (or heritable) fragile sites only in less than 5% of human population (Schwartz et al., 2006). Under particular in vitro conditions, the chromosomal fragility is displayed by broken chromatids (usually both), which results in forming acentric fragments, deleted chromosomes, triradial figures and similar abnormalities that occur on metaphase spreads (Sutherland, 1979). Further division of fragile sites is therefore provided by their inducing factors. Common fragile sites are detectable after aphidicolin, 5-azacytidine or 5-bromodeoxyuridine (BrdU) treatment. Rare fragile sites distinguish folate sensitive, distamycin A-inducible and BrdU-requiring groups (Handt et al., 2000a). The two distamycin A-inducible ones are also BrdU inducible (Sutherland, 2003).

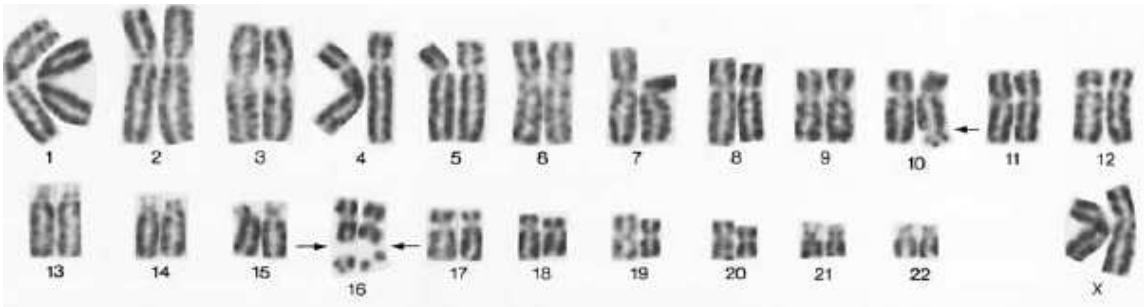

Figure 1. Karyotype of a woman heterozygous for FRA10B (arrow) and homozygous for FRA16B (arrows) after cell treatment with berenil (derivate of distamycin A). Adopted from Felbor et al. (2003).

Inducer	Fragile site	Chromosomal location	Molecular cloning	Inducer	Fragile site	Chromosomal location	Molecular cloning
Common fragile sites							
Aphidicolin	FRA1A	1p36		Aphidicolin	FRA8D	8q24.3	
	FRA1B	1p32			FRA9D	9q22.1	
	FRA1C	1p31.2			FRA9E	9q32	+
	FRA1D	1p22			FRA10D	10q22.1	
	FRA1E	1p21.2			FRA10E	10q25.2	
	FRA1F	1q21			FRA10F	10q26.1	
	FRA1G	1q25.1			FRA10G	10q11.2	
	FRA1I	1q44			FRA11C	11p15.1	
	FRA1K	1q31			FRA11D	11p14.2	
	FRA1L	1p31			FRA11E	11p13	
	FRA2C	2p24.2			FRA11F	11q14.2	
	FRA2D	2p16.2			FRA11G	11q23.3	
	FRA2E	2p13			FRA11H	11q13	
	FRA2F	2q21.3			FRA12B	12q21.3	
	FRA2G	2q31	+		FRA12E	12q24	
	FRA2H	2q32.1			FRA13A	13q13.2	
	FRA2I	2q33			FRA13C	13q21.2	
	FRA2J	2q37.3			FRA13D	13q32	
	FRA3A	3p24.2			FRA14B	14q23	
	FRA3B	3p14.2	+		FRA14C	14q24.1	
	FRA3C	3q27			FRA15A	15q22	
	FRA3D	3q25			FRA16C	16q22.1	
	FRA4A	4p16.1			FRA16D	16q23.2	+
	FRA4C	4q31.1			FRA17B	17q23.1	
	FRA4D	4p15			FRA18A	18q12.2	
	FRA4F[a]	4q22	+		FRA18B	18q21.3	
	FRA5C	5q31.1			FRA20B	20p12.2	
	FRA5D	5q15			FRA22B	22q12.2	
	FRA5E	5p14			FRAXB	Xp22.31	+
	FRA5F	5q21			FRAXC	Xq22.1	
	FRA6B	6p25.1		Total = 77	FRAXD	Xq27.2	
	FRA6C	6p22.2					
	FRA6E	6q26	+	BrdU	FRA4B	4q12	
	FRA6F	6q21	+		FRA5A	5p13	
	FRA6G	6q15			FRA5B	5q15	
	FRA7B	7p22			FRA6D	6q13	
	FRA7C	7p14.2			FRA9C	9p21	
	FRA7D	7p13			FRA10C	10q21	
	FRA7E	7q21.2	+	Total = 7	FRA13B	13q21	
	FRA7F	7q22					
	FRA7G	7q31.2	+	5-Azacytidine	FRA1H	1q42	
	FRA7H	7q32.3	+		FRA1J	1q12	
	FRA7I	7q36	+		FRA9F	9q12	
	FRA7J	7q11		Total = 4	FRA19A	19q13	
	FRA8B	8q22.1					
	FRA8C	8q24.1	+	Unclassified	FRA4E	4q27	
Rare fragile sites							
Folate deficiency	FRA1M	1p21.3		Folate deficiency	FRA19B	19p13	
	FRA2A	2q11.2			FRA20A	20p11.23	
	FRA2B	2q13			FRA22A	22q13	
	FRA2K	2q22.3			FRAXA	Xq27.3	+
	FRA2L[b]	2p11.2			FRAXE	Xq28	+
	FRA5G	5q35		Total = 24	FRAXF	Xq28	+
	FRA6A	6p23					
	FRA7A	7p11.2		Distamycin A only	FRA8E	8q24.1	
	FRA8A	8q22.3			FRA11I	11p15.1	
	FRA9A	9p21		Total = 3	FRA16E	16p12.1	
	FRA9B	9q32					
	FRA10A	10q23.3	+	Distamycin A and BrdU	FRA16B	16q22.1	+
	FRA11A	11q13.3		Total = 2	FRA17A	17p12	
	FRA11B	11q23.3	+				
	FRA12A	12q13.1		BrdU	FRA10B	10q25.2	+
	FRA12D	12q24.13		Total = 2	FRA12C	12q24.2	
	FRA16A	16p13.11	+				
	FRA18C	18q22.1		Unclassified	FRA8F	8q13	

Table 1. (previous page) The list of all fragile sites published in Schwartz et al.(2006)

2.2. General characteristics of rare fragile sites

Cytogenetically, fragile site is a part of chromatin, which ceases to compact for mitosis. While the normal regions of DNA finish replication before metaphase, the fragile regions seem to remain unreplicated or even single-stranded. This can result in forming of chromosomal breaks in a certain percentage of cells. Even though they might manifest spontaneously, their inducers cause replication stress, further enhancing their expression (Sutherland, 2003). Furthermore, the expressed rare fragile sites were found to expel nucleosomes in the presence of their inducers. This attribute was strongly correlated with a length of the sequence (Wang et al., 1996; Hsu and Wang, 2002). It was therefore possible to assess the chromosomal constrictions at fragile sites to be the consequences of perturbed nucleosome formation.

Some fragile sites follow Mendelian inheritance (FRA16B, FRA10B), while others do not: FRAXA is transmitted differentially by males and females (Sutherland, 2003).

Rare fragile sites consist of different copy numbers of certain repeat sequences. Thus, they were generally determined as subjects of dynamic mutation process (Yu et al., 1997). Their increasing copy number positively correlates with the rate of their instability, both somatic and intergenerational, and severity of disease, if they are associated with any (Richards and Sutherland, 1997). The stability of fragile sites is also influenced by the sequences flanking the expanded alleles (Sutherland and Richards, 1995). However, the folate sensitive fragile sites show higher instability, which may be due to their shorter repeat sequences that are more prone to replication slippage than their longer counterparts (Sutherland and Richards, 1999).

2.3. Folate sensitive rare fragile sites

The majority of rare fragile sites is induced either by folate or thymidine deficiency in cell culture media, folate metabolism inhibitors (e.g. methotrexate) or dTTP synthesis inhibitors. Their frequency also rises with increasing pH, which is useful for acquisition of maximal in vitro expression (Sutherland, 1979). Seven of them were positionally cloned and found to be expanded CCG microsatellite repeats, showing polymorphism in the copy number in individuals (Schwartz et al., 2006; Winnepenninckx et al., 2007). Alleles express fragility

after reaching the threshold of 100-1000 copies (Pearson and Sinden, 1998). Lower copy numbers are not cytogenetically visible, although these of 50-1000 copies in length are thought to be premutations, which expand into full mutations during oogenesis, but not during spermatogenesis (Sutherland et al., 1999). Expanded alleles have the contiguous CpG islands hypermethylated, which seems to be the consequence rather than the cause of the mutation (Nancarrow et al., 1994). Short nonfragile sequences are stabilized by interruptions, whereas fragile alleles or premutations contain long stretches of perfect repeats, thus being prone to further expansion (Handt et al., 2000a)

CCG trinucleotide repeats can form non-B DNA secondary structures (Figure 2): intra-strand hairpin structures, slipped strand DNA and tetrahelical structures (reviewed in Pearson and Sinden, 1998). Stability of such an organization of these sequences depends on their length and purity. Hairpins can lower the difference between energy levels of normal and slipped state, thus providing stability for a strand slippage, which is necessary for the dynamic mutation process (Gacy et al., 1995). Tetrahelical structures may occur immediately when CCG-rich DNA is unpaired and contribute to the perturbation of DNA replication. Obstruction of polymerase progression would further facilitate strand slippage on repeated sequences (Usdin and Woodford, 1995).

Only three members of folate sensitive group are generally considered to be clinically significant, FRAXA, FRAXE, and FRA11B. FRAXA, which localizes to Xq27.3, is associated with fragile X syndrome. At its position in normal chromosome, there are 5 to 55 copies of CCG repeat, which increase of about 200 copies in premutation carriers. Full mutation consists of more than 230 copies of CCG repeat with adjacent highly methylated CpG sequences. Female carriers of premutation may rarely display the fragile X, which is probably due to the hypermethylation of this chromosome that is necessary for expression of the fragile site. Fragile X syndrome itself is caused by transcriptional silencing of the FMR1 gene, in whose 5´ untranslated region a repeat expansion appears. Expansion of FRAXE repeats is followed by non-specific X-linked mental retardation (Sutherland, 2003). Third fragile site on X chromosome, FRAXF, have never been documented to cause any clinical problem. Rare fragile sites on X chromosome are cytogenetically indistinguishable, thus molecular analysis has to be done to determine a potential pathological significance (Sutherland and Baker, 2000). FRA11B was demonstrated to map within 5´ UTR of the CBL2 proto-oncogene. Although it had been previously associated with Jacobsen syndrome, the deletion breakpoint found in some patients was proposed to localize proximally to this fragile site (Jones et al., 1995). Recently, FRA12A was cloned and demonstrated to contain the

DIP2B gene, whose down-regulation may be associated with mental retardation (Winnepenninckx et al., 2007).

Interestingly, folate sensitive type of fragile regions has not been observed in species other than humans (Sutherland and Richards, 1999). The homozygous carriers of folate sensitive fragile sites on autosomal chromosomes have never been reported and the heterozygotes comprise only 1% of the human population. It was therefore considered that homozygosity may lead to spontaneous abortion or risk of defects of genetic origin in children with heterozygous parents (Sutherland and Baker, 2000).

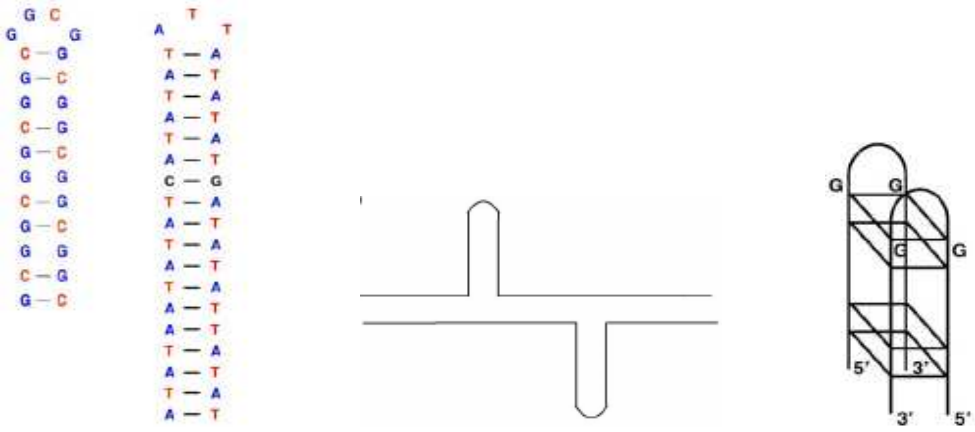

Figure 2. Unusual secondary structures formed by fragile sequences: intra-strand hairpins (left), slipped-strand DNA (middle) and tetrahelical structures (right). Adopted from Schwartz et al. (2006)

2.4. Non-folate sensitive rare fragile sites

These fragile sites are present in 6-8% of human population (Sutherland and Baker, 2000) and are divided in the two groups mentioned above. Distamycin A inducible group has 5 members to date. Their expression is caused by specific compounds that bind to the minor groove of DNA, which also comprises Hoechst 33258, netropsin and berenil (Sutherland, 2003).

Second, BrdU requiring group, is represented by only two members. Their inducer can be incorporated into the DNA during replication instead of thymine. It is used in concentration that only partially inhibits replication (Schwartz et al., 2006).

Propensity of these fragile sites to form non-B DNA structures has not been examined yet. But since this attribute was proposed to be necessary for repeat expansion (Gacy et al., 1995), it was hypothesised, that all types of rare fragile sites shared this property (Hewett et al., 1998).

Two non-folate sensitive rare fragile sites have been cloned, both containing AT-rich minisatellite repeats, which expand under conditions required (Schwartz et al., 2006). Compared to folate sensitive fragile sites, expanded alleles of these fragile sites are up to 20 times longer (Sutherland et al., 1998). They were demonstrated to be late replicating, although FRA10B showed delay only on the distal side of the expanded allele. However, delayed replication may contribute to fragility, but it is probably not a sufficient condition for expression of fragile site, because gaps are displayed only by a fraction of cells (Handt et al., 2000b).

The most common rare fragile site, FRA16B, is induced either by minor-groove binding chemicals or by BrdU (Felbor et al., 2003). When induced with berenil, it occurred in almost 100% of metaphases (Sutherland and Richards, 1995). The homozygotes have been found to be normal, without any gene affected, at least no essential one (Hocking et al., 1999). The probands of this study were also heterozygous for another rare fragile site. However, possible relation between both aberrations has not been examined, as in other cases of this phenomenon (Sutherland and Baker, 2003). FRA16B was first determined as an allelic expansion of 33-base pair consensus sequence located at 16q22.1, proposed to be a region of genomic instability. Normal alleles ranged from 7 to 12 copies, rising up to 2000 upon amplification (Yu et al., 1997). Then a novel 35-bp minisatellite repeat was described in a Japanese carrier, differing in insertion of two nucleotides from the previous one (Figure 3) (Yamauchi et al., 2000). This study also suggested that the length of the expanded region might be more significant for cytogenetic expression of this fragile site than its AT content.

5‘ - ATA TAT TAT ATA TTA TAT CTA ATA A TAT ATA TA - 3‘
5‘ - ATA TAT TAT ATA TTA TAT CTA ATA A TAT ATC TA – 3‘
5‘ - ATA TAT TAT ATA TTG TAT CTA ATA A**TA** TAT ATC TA – 3‘
5‘ - ATA TAT TAT ACA TTG TAT CTA ATA A**TA** TAT ATC TA – 3‘

Figure 3. Repeat motif of FRA16B reported by Yu et al. (1997) (first and second row) and by Yamanauchi et al. (2000) (third and fourth row). Inserted nucleotides are shown in bold.

FRA10B can be induced only by BrdU (or BrdC) and has not been found to cause any disease (Sutherland, 2003). Homozygous children reported in early study were phenotypically normal (Sutherland, 1981). This fragile site is located at 10q25.2. FRA10B repeats vary between 16 and 52 bp in length (in single individual). Four groups of alleles were designated: small normal (<1 kb), from which progressively arise intermediate (1-4 kb), large normal (4-5 kb) and expressing one (>5 kb). This finding is consistent with the dynamic mutation process of fragile site formation. Surprisingly, sequences of these two fragile sites share 26 identical bases (Hewett et al., 1998).

2.5. Rare fragile sites and evolution

The evolutionary biologists have long been considered existence of chromosomal regions that have been repeatedly used in genome rearrangements. Recent findings (Ruiz-Herrera et al., 2006) confirmed a non-uniform distribution of these evolutionary 'hot spots', some of which are conserved during the chromosome evolution. The evolutionary breakpoints were demonstrated to span tandem repeats and fragile sites, although this association was statistically significant just for the rare ones. This study proposed human genome to be a composition of fragile regions, conserved in mammals and disposed to reorganization, and regions lying between them without showing evolutionary plasticity.

2.6. Common properties of rare and common fragile sites

Both types of fragile sites have the same cytogenetical manifestation, thus there have been many attempts to find out their possible common characteristics on molecular level (Schwartz et al., 2006).

Above all, fragile sites share perturbed or late replication in terms of their induction mode. Then, all the classes form stable secondary DNA structures, which may obstruct the replication elongation. Finally, they share a cytogenetical manifestation.

Common fragile sites have not been elicited to comprise any expanded repeats, but interrupted shorter repeats. Thus, the fragility of these sequences may be accounted on their AT-richness and flexibility (Ried et al., 2000). Stretches of AT-rich islands at common fragile sites and repeat motifs of FRA10B and FRA16B were found to be highly similar. These two rare fragile sites also showed flexibility comparable to common fragile sites. Normal alleles of FRA16B and FRA10B stretch the same regions on human genome as common fragile sites

FRA16C and FRA10E, respectively. Furthermore, some of the rare fragile sites, which have not been cloned yet, span the same chromosomal bands as common fragile sites inducible by aphidicolin treatment. It was therefore considered that common fragile sites might expand into rare fragile site (Zlotorynski et al., 2003). This study also proposed that fragility would result from different replication progression between fragile and nonfragile regions.

3. Involvement of rare fragile sites in cancer

3.1. Fragile sites and double strand breaks

Propensity of fragile sites to form hairpins is the possible reason for their capability to delay replication because it takes some time to get over these structures. Under further replication stress, the fragile regions entirely halt replication (Arlt et al., 2003). Abnormal stimulation of cell proliferation in precancerous stages was suggested to cause such replication stress (Gorgoulis et al., 2005). The perturbed replication may be followed by replication fork collapse and formation of DNA double strand breaks (DSBs) (Lundin et al., 2002), which are one of the most dangerous forms of DNA damage. They appear by simultaneous breakage of both DNA strands at sites that are close enough to be impossible to be kept together (Jackson, 2002). This event switches signal transduction pathways to repair these lesions on DNA. When these pathways fail, either cell death or chromosomal rearrangements occur (Khanna and Jackson, 2001).

As mentioned above, inducers of fragility can inhibit the progression of replication forks. The low concentration of the replication inhibitor, which only slows the process, led to formation of DSBs in some genomic regions, whereas the higher concentration, followed by replication arrest, caused lesions throughout the entire genome (Schwartz et al., 2005).

The main DSB repair pathways are homologous recombination (HR) and nonhomologous end joining (NHEJ). They function in partly overlapping manner. The replication inhibitors arrest cell cycle in late G1 and S phase. Stalled replication forks can result in forming of single strand nicks, followed by DSBs. The stimulation of HR is enhanced by prolonged DNA replication block and increasing number of these lesions. It proceeds mostly by gene conversion. While HR occurs mostly in the late phase, NHEJ would repair DNA damage in the early response (Saintigny et al., 2001). NHEJ acts by simple ligation of two free DNA ends without requirement of their homology (Khanna and Jackson, 2001). The roles of these two pathways may be also cell cycle-dependent, NHEJ being engaged in all phases, whereas HR mostly in late S/G2 (Rothkamm et al., 2003). More recently, HR was proposed to be a secondary event even in terms of repair action itself, DSBs being firstly fixed by NHEJ due to higher safety of the process (Iliakis et al., 2004). However, this study inquired into ionizing radiation induced lesions, thus considering that the selection of the repair pathway may depend on the inducing factor. This is in agreement with previous findings (Rothkamm et al., 2003), which demonstrated the preferential role of HR in regeneration of DSBs produced by treatment with aphidicolin. Furthermore, HR is also the

sole process in restoration of slowed replication forks without presence of DSBs (Lundin et al., 2002).

Once broken, neither fragile chromosomes, nor cut fragments seem to restore their telomeres (Villa et al., 1997). Thus, they are prone to forming aberrant structures that may occur through breakage-fusion-bridge (BFB) cycles (Figure 4). Fusion of chromosomes with broken ends or shortened telomeres results in formation of dicentric chromosome, followed by new breakage at anaphase. This event generates chromosome with inverted duplication of terminal sequences, which would enter another cycle, until it gains telomeric sequences (McClintock, 1942). The BFB mechanism was also suggested to act towards the amplification of certain genomic regions necessary for successful cancer development (e.g. oncogenes) (Albertson, 2006). Expression of fragile sites was demonstrated to be the major cause of this event at its early stages. The distance between the fragile sites involved in amplification determines the length of the amplified region. When the amplicon is accumulated in sufficient amount, the breaks in following BSB cycles seem to appear at random positions (Coquelle et al., 1997).

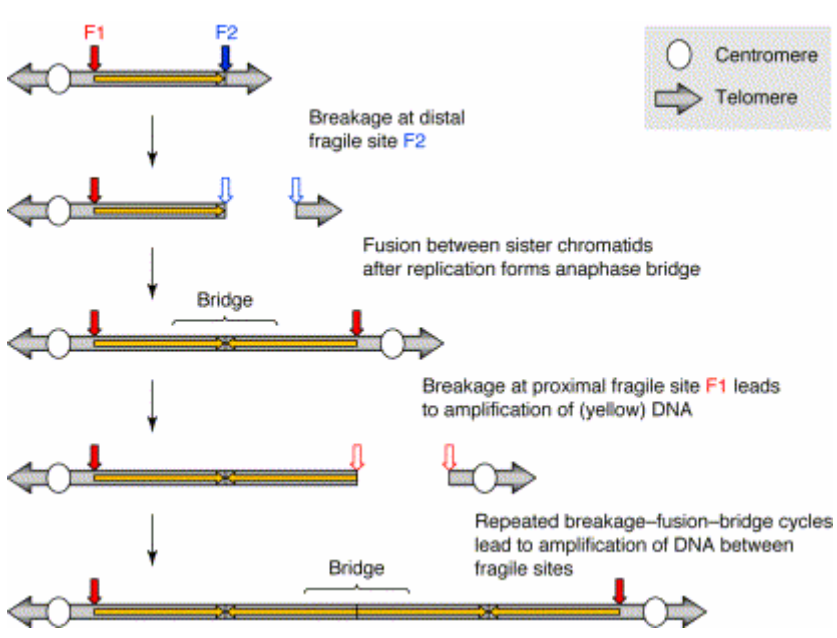

Figure 4. Breakage-fusion-bridge cycles, model with two fragile sites at one chromosomal arm. See text for details. Adopted from Sutherland et al. (1998).

Hypoxia was proposed to induce fragile sites in vivo and to cause fusion of acentric extrachromosomal molecules called double minutes (DMs), which often contain amplified oncogenes. Moreover, these were demonstrated to reintegrate into fragile sites, resulting in formation of chromosomal expansions visible as homogenously stained regions (HSRs) that are frequently observed in solid tumours. Repeated induction of fragile sites was shown to produce large marker chromosomes with accumulated genes (Coquelle et al., 1998).

Taken together, the results of these two French studies mentioned above clearly show that fragile sites are associated with both extrachromosomal and intrachromosomal amplification of oncogenes.

3.2. Rare fragile sites in leukemic patients

Chromosomal rearrangements associated with fragile site expression were considered to be the cause of neoplastic transformation. This was especially examined in various cases of leukemia. An early study (Mules et al., 1989) investigated possible hereditary predisposition to cancer in relatives of patients with lymphoid leukemia, who have chromosomal rearrangements at rare fragile sites. Although the male relatives of the cases in older age groups displayed tendency to lung cancer, it was not statistically significant. Furthermore, hereditary proneness to cancer might show earlier age at onset, but younger relatives were not affected. Thus, cancer predisposition in these families due to chromosomal aberrations caused by rare fragile sites was not proved. However, with the numbers of relatives in this study, possible slight tendency to cancer was undetectable.

Another study examined a single family, which inherited autosomal dominant acute myelogenous leukemia (Horwitz et al., 1997). Even though LOD score (logarithm of odds; a statistical test used for the analysis of the genetic linkage between traits or markers in pedigrees, i.e. to determine the probability of recombination) provided linkage to 16q22 locus, expansion of FRA16B as a cause of leukemia in this family was excluded.

Chromosomal aberrations were demonstrated to occur non-randomly in cells of patients with chronic myeloid leukemia (Fundia et al., 2000). This report proposed chromosomal instability to appear as a consequence of clastogenic environment in cancer cells, thus being a demonstration of a neoplastic process.

Cells from patients with chronic lymphocytic leukemia frequently contain chromosome 11q deletions. Since clusters of CCG repeats including those of FRA11B are known to localize on distal region of longer arm of the chromosome 11, they were examined to be

involved in progression of the disease (Auer et al., 2001). Although chromosomal breakpoints were situated on other CCG rich regions, minimal deleted region spans FRA11B. It was therefore suggested that there might exist previously unrevealed fragile sites or that unstable repeats of fragile site would influence stability of the region far away from its own position. Moreover, placed in deleted region, contribution of FRA11B to neoplastic development is hard to detect.

Possible implications of fragile sites in cancer may also occur through epigenetic mechanisms. Genes of non-coding RNAs, called microRNAs (miR genes) were found to frequently coincide with fragile sites and genomic regions associated with cancer (Calin et al., 2004). MicroRNAs usually regulate expression of other genes through translation blockade (Huppi et al., 2007). Two miRs were previously demonstrated to be down-regulated or deleted in most of chronic lymphocytic leukemia cases (Calin et al., 2002).

3.3. Possible role of rare fragile sites in anticancer therapy

The AT-rich minisatellite of fragile site FRA16B was proposed to serve as nuclear matrix attachment region (MAR) in leukemic cells, whereas normal alleles were found in the loop DNA of normal cells (Jackson et al., 2003). Since MARs are implicated in initiation of DNA replication, they are crucial for tumour cells. Thus, these sequences were demonstrated to be preferentially targeted by potent antitumour drugs like bizelesin, which give rise to high number of lesions on AT-rich DNA (Woynarowski et al., 2001). Given the difference in composition between the normal and the expanded FRA16B, this might be beneficial for the efficient removal of cancerous cells. However, not all MARs described to date are AT islands and vice versa (Woynarowski, 2004).

Interestingly, one of the inducers or this fragile site, BrdU, was demonstrated to fortify the binding of MARs to nuclear matrix. This structural alteration would change expression of genes in the vicinity of MAR, as those associated with cellular senescence (Ogino et al., 2002).

3.4. Fragile sites as consequences of failure of the genome maintenance mechanisms

Reports on individuals with more than one fragile site offer a possibility that some trans-acting agent, which brings about one fragile sequence expansion, may act on more regions prone to copy-number alterations (Sutherland and Baker, 2003). Lack of proteins involved in

genome maintenance was proved to induce common fragile site expression (Casper et al., 2002, Musio et al., 2004). Since cytogenetical appearance and defective replication are shared by both types of fragile sites, it might be some mutation in members of signal transduction pathways, cell cycle checkpoints or other mechanisms comprised in genome surveillance, which is responsible for or contribute to fragility. This is in concert with finding of chromosomal instability in Seckel syndrome, a rare autosomal recessive disorder, which is manifested by dwarfism, mental retardation, microcephaly, micrognathia and other facial malforations. Clinical features of this disease are equivalent to those of Nijmegen breakage syndrome and LIG4 syndrome, both of which being associated with altered DNA damage response. Seckel syndrome was proposed to be an outcome of low level of ataxia-teleangiectasia and Rad3-related protein (ATR), which is sufficient to prevent cell death, but deficient in case of replication stress (Casper at al., 2004).

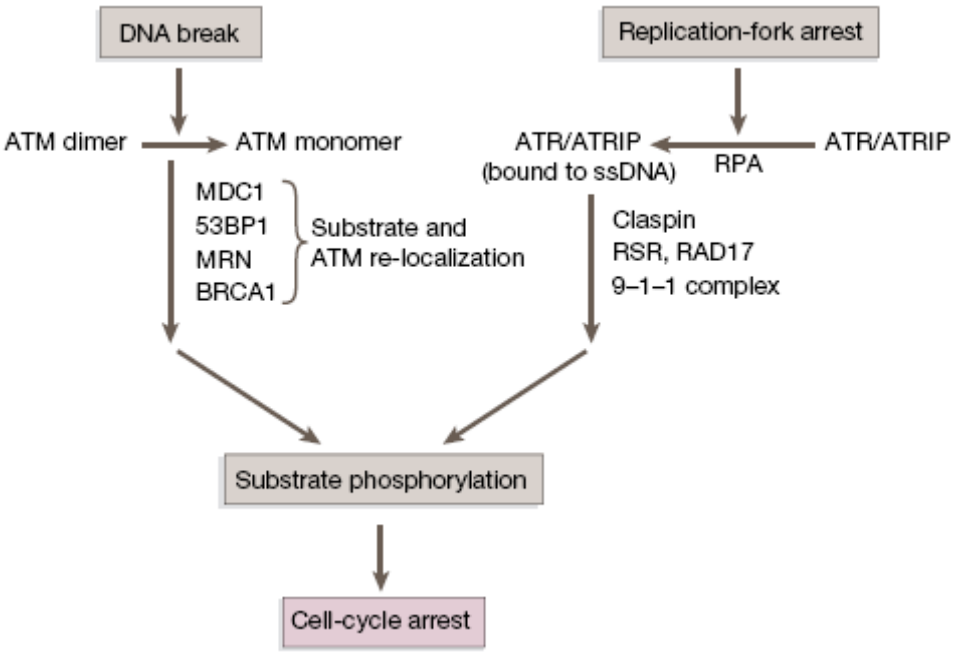

Figure 5. DNA DSBs lead primarily to ATM activation, whereas ssDNA attract ATR-ATRIP complex. Both kinases then phosphorylate their respective downstream targets, including those that establish and sustain cell-cycle arrest. Adopted from Kastan and Bartek (2004).

On the way from DNA damage to its repair, there is a group of less or more important proteins employed in various steps of the process: sensors that recognize the lesion, kinases of transduction cascade and effector proteins, which provide DNA repair, cell cycle arrest, or apoptosis (Khanna and Jackson, 2001). The regions of single stranded DNA (ssDNA), which appear at stalled replication forks (Figure 5), DSBs or sites of other types of DNA damage (e.g. mismatch repair), are recognized and covered by replication protein A (RPA) (Namiki and Zou, 2006). These protein structures attract ATRIP (ATR interacting protein) (Zou and Elledge, 2003), which may also interact with ssDNA itself, but this interaction seems to be of secondary significance (Namiki and Zou, 2006). ATRIP is phosphorylated by ATR and these two proteins create a stable complex in human cells, which appears as intranuclear foci at sites of DNA damage. Furthermore, their expression is mutually dependent (Cortez et al., 2001) and loss of ATR leads to embryonic lethality at early stage of the development, that was caused by chromosomal fragmentation (Brown and Baltimore, 2000). RPA is necessary not only for formation of ATR-ATRIP nuclear foci at sites of DNA damage, but also for the activation of downstream targets of ATR, which was demonstrated for Chk1 (checkpoint kinase 1) and Rad17. Besides ATR-ATRIP, Rad17 and Rad9-Rad1-Hus1 complexes also contribute to the recognition of lesions on DNA (Zou and Elledge, 2003).

ATR was previously thought to exist in a stably active form, which only relocalizes to actual DNA lesions. Recent findings demonstrated that it is activated by interaction with TopBP1 (Figure 6, left), which would alter the conformation of ATR-ATRIP complex. The transient character of this interaction allows ATR to pursue its protective function in a dynamic and efficient manner (Kumagai et al., 2006). ATR is consequently capable to directly phosphorylate majority of its substrates with exception of Chk1 (Liu et al., 2000), which is also regulated by Claspin (Liu et al., 2006). Activated Chk1 initiates cell cycle arrest by inhibition of Cdc25C (Sanchez et al., 1997), thus preventing mitotic entry. Phosphatase Cdc25A, which also promote cell cycle progression, was demonstrated to be degraded by ubiquitination in response to DNA damage (Mailand et al., 2000).

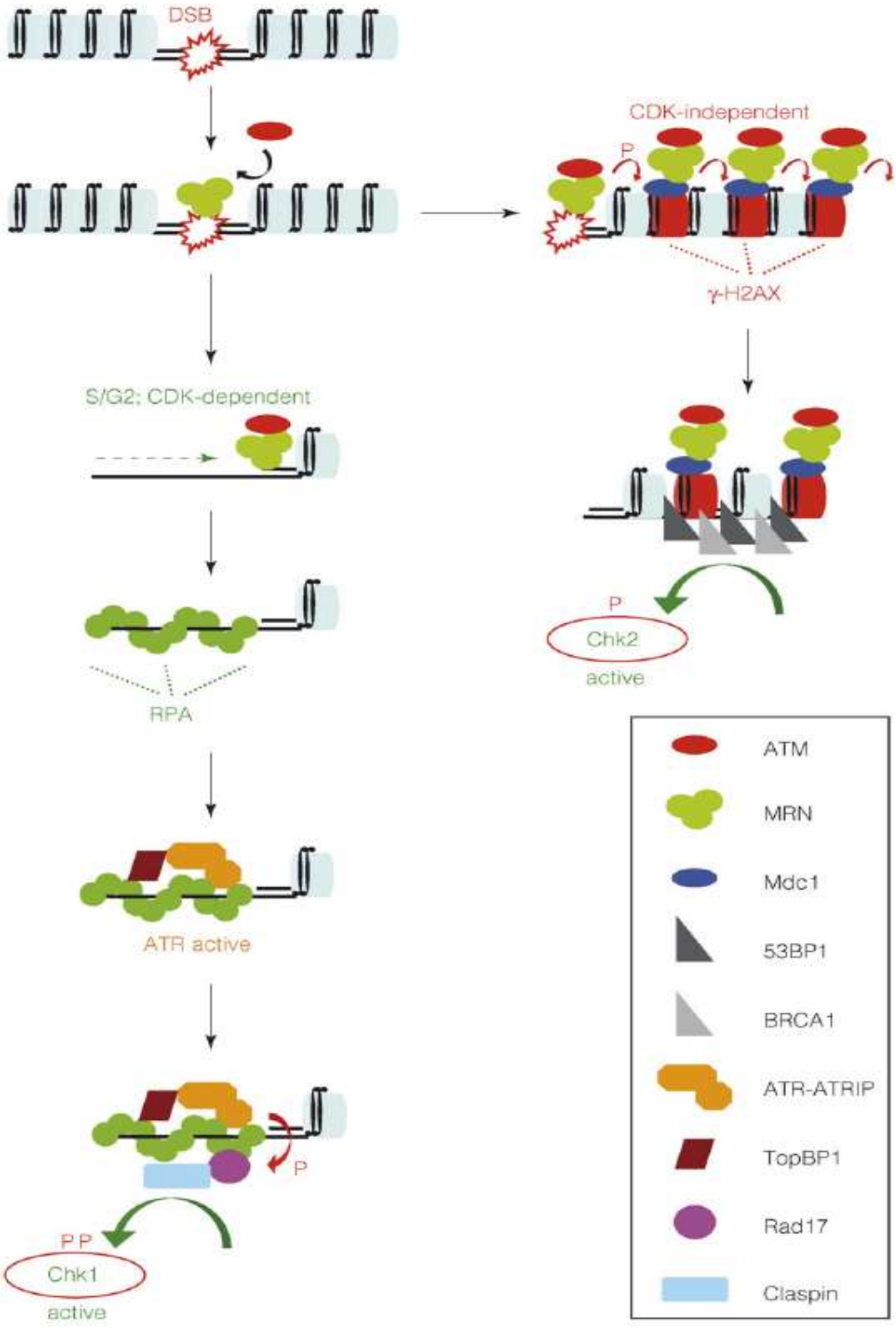

Figure 6. Both ATR (left) and ATM (right) are required for the cell response to DNA DSBs. See text for details. Adopted from Bartek and Lukas (2007).

Another kinase crucial for DNA damage response is ataxia teleangiectasia mutated protein (ATM), which is particularly involved in cellular response to DSBs. In normal, undamaged cells it exists in dimeric or multimeric form, which is dissoluted by autophosphorylation in the presence of DNA damage (Figure 5). Active monomers subsequently phosphorylate substrates (Bakkenist and Kastan, 2003). Another research group proposed that ATM activation is provided by MRN (Mre11-Rad50-Nbs1) complex, which senses DSBs and unwinds DNA ends (Lee and Paull, 2005). This study did not confirm the necessity of ATM autophosphorylation. However, ATM is recruited to DSBs by interaction with C terminus of Nbs1 (Falck et al., 2005).ATM targets various substrates as p53 by phosphorylation at serine 15 (Banin et al., 1998, Canman et al., 1998), Chk2 (chekpoint kinase 2), or H2AX, which is also phosphorylated by ATR at sites of stalled replication forks (Ward and Chen, 2001). Phosphorylated form of H2AX, γH2AX, then binds MDC1 (mediator of DNA damage checkpoint protein 1) and this interaction is necessary for formation of 53BP1 (p53 binding protein 1), Nbs1 (Nijmegen breakage syndrome 1) and active ATM foci at sites of damaged DNA (Figure 6, right). ATM is also required to maintain γH2AX in its activated form (Stucki et al., 2005).

In response to DSBs in S and G2 phases, both ATM and ATR are required (Figure 6). ATM triggers Mre11 nuclease activity, which subsequently generate stretches of ssDNA that are coated with RPA. These intermediates attract ATR, whose activated downstream effector Chk1 cooperates with ATM-activated Chk2 in cell cycle arrest (Jazayeri et al., 2006). ATM and ATR are both members of the phosphoinositide-3-kinase-related protein kinase (PIKK) family and they share some important targets as p53 or BRCA1 (breast cancer 1) (Tibbetts et al., 2000), which are both tumour suppressors and their loss results in malignancy (Niida and Nakanishi, 2006). The PIKK family also comprises DNAPKcs (DNA-dependent protein kinase catalytic subunit). This enzyme interacts with C terminus of Ku80 to form the DNA-PK complex, which is involved in NHEJ DSBs repair (Falck et al, 2005). The DNA damage repair is provided by HR and NHEJ, which were discussed above.

Interestingly, some large common fragile sites span genes involved in cellular response to various types of stress (including replication stress), as FRA15A, which contain retinoic acid receptor-related orphan receptor alpha (RORA) gene (Zhu et al., 2006). Lying in highly unstable regions, these genes are frequently deleted during neoplastic development and at least two of them have been demonstrated to play a role in tumour suppression. However, their exact function in carcinogenesis remains to be elucidated (Smith et al., 2007).

4. Conclusion

Fragile sites are unstable regions of mammalian genomes. According to Schwartz et al. (2006), there are 89 common and 32 rare fragile sites in human genome. Indeed, not all of them have been revealed to date. Although some rare fragile sites have been characterized at the molecular level, the connection between the certain structure and respective inducing factors of all types of fragile alleles remains elusive (Handt et al., 2000a). It is also still unclear, which factors are responsible for expression of fragility in vivo (Richards, 2001).

Some of rare fragile sites were previously associated with alterations in gene expression resulting in mental retardation (Sutherland, 2003). However, in terms of cell cycle progression, their major importance is their ability to cause replication stalling or even DNA DSBs. Such lesions on DNA require quick and efficient repair. It was recently reported, that at early stages of neoplastic transformation, pathways of the DNA damage response are activated. In developed malignant tumours, these mechanisms were diminished (Bartkova et al., 2005). The authors of this study therefore proposed, that mutations causing defects in DNA damage signalling and repair are the steps to genomic instability associated with development of cancer.

DNA damage response was recently proposed as a necessary component of replication and processing of human telomeres (Verdun and Karlseder, 2006). Since telomere attrition and genomic instability are both hallmarks of neoplastic transformation, it is tempting to speculate that fragile sites might indicate mutations in repair pathways or somehow reflect precancerous cell environment. The demonstration of fragile site induction by hypoxic conditions in vitro (Coquelle et al., 1998) further supports this idea. Moreover, hypoxia may cause a selective pressure for transformed cells, which lost functional p53 or other components of checkpoint control mechanisms (Hammond et al., 2002).

Although fragile sites are usually placed to regions, which are deleted in cancer, FRA16B was found in its expanded form in leukemic and colon carcinoma cell lines (Jackson et al., 2003). Possible contribution of fragile sites to carcinogenesis by oncogene amplification was demonstrated only in vitro in Chinese hamster cell line (Coquelle et al., 1997). However, if fragile sites has causal role in neoplastic development or they are just its consequences requires further investigation.

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