Progress in Biological Sciences

Vol. 4, Number 2, Summer / Autumn 2014/261-273

Apoptotic protease-activating factor 1 (Apaf-1) as a liable gene for spontaneous mutations in vitro

Received: 10 September, 2014; Accepted: 30 October, 2014

Taha Azad[1,3], Amin Tashakor[1,3], Mina Ghahremani[2], Roohullah Hemmati[1], Farangis Ataei[1], Saman Hosseinkhani[1]*

1. Department of Biochemistry, Faculty of Biological Sciences, Tarbiat Modares University, Tehran, Iran.
2. Department of Plant Biology, Faculty of Biological Sciences, Tarbiat Modares University, Tehran, Iran.
3. These authors contributed equally to this work

ABSTRACT

The apoptotic protease-activating factor 1 (Apaf-1) receives the death signal in the intrinsic or mitochondrial pathway of apoptosis. Upon the releasing of cytochrome c from the intermembrane space of mitochondria and binding to Apaf-1 molecules, a heptameric apoptosome complex is formed and triggers the downstream cascade of caspases. Here, for the first time we present spontaneous mutations and recombinations of the Apaf-1 gene and its neighbouring sequences. We sequence 48 colonies containing pcDNA3.1 vector with Nluc/Apaf1 and Cluc/Apaf1 obtained through the quick-change site-directed mutagenesis method, transforming to DH5-α and XL10-Gold at two temperatures, 18 and 37ºC. In 21 of these cases, we found 38 different mutations. Our data suggest that there is a direct relationship between bacterial incubation temperatures and the number of unwanted spontaneous mutations. During our experiment we found that the Apaf-1 gene is much less susceptible to spontaneous mutations when it is transformed into XL10-Gold at 18ºC. In contrast, a large number of spontaneous mutations were found when the gene of interest transformed into DH5α at 37ºC.

Keywords: apoptosis, apoptotic protease-activating factor 1 (Apaf-1), cancer, spontaneous mutations, WD-40 repeats.

* **Corresponding author:** saman_h@modares.ac.ir

Introduction

Apoptosis, programmed cell death, has a key role in many important biological phenomena. In metazoan, apoptosis shapes tissues during development and maintains the number of cells at an appropriate level. In mitochondrial pathway of apoptosis (intrinsic), the main soluble receptor is Apoptotic protease-activating factor 1 (Apaf-1) (1). Cytochrome *c* proteins, which are released from the intermembrane space of mitochondria into the cytosol in response to intracellular stress signals, bind to Apaf-1 monomers and cause oligomerization and apoptosome machine formation (2). Apaf-1 is a 130 KD protein with three functional domains: the Caspase Recruitment Domain (CARD), located at the N-terminal, the Nucleotide-binding and Oligomerization Domain (NOD), and the C-terminal WD40-repeat domain (3). The CARD function binds the caspase-9 by homotypic CARD-CARD interactions and brings it to the apoptosome complex for activation (4). The NOD, also referred to as the NB-ARC domain, comprises three subdomains located between the CARD and WD-40 repeats. According to structural studies it consists of an ATPase subdomain homologous to similar domains found in a large family of ATPases, the AAA+ family, followed by a winged-helix subdomain and a superhelical subdomain (5). Because the NB-ARC domain is located at the centre of Apaf-1, it usually is regarded as an important part that mediates oligomerization and apoptosome formation (6). According to the various deletion mutants generated in a study which investigated the behaviour of various truncated Apaf-1, without the NB-ARC domain apoptosome complex cannot form; two domains, the CARD and the NB-ARC, are necessary for apoptosome formation (7). WD-40 repeats were organized into two domains with a seven- and eight-blade beta propeller. In the absence of cytochrome *c*, WD-40 repeats lock Apaf-1; conversely, by attachment of cytochrome *c* to this part of Apaf-1, it switches on and oligomerizes to apoptosome complex. With this in mind, truncated Apaf-1 lacking WD-40 repeats shows autoassembly and procaspase-9 activation without any specific response to releasing cytochrome *c* (7, 8).

In our previous study, we presented a novel whole-cell biosensor to detect the early stages of programmed cell death based on Apaf-1 oligomerization and apoptosome formation using the split luciferase complementation strategy. For this, the amino (1–416 amino acids) and carboxy fragments (395–550 amino acids) of firefly luciferase were separately fused to amino-terminal of Apaf-1. Juxtaposition of Apaf-1 monomers and apoptosome formation were detected by emission of luminescence signal (Fig. 1). This research made the real-time and accurate study of apoptosome formation and caspase-3/7 activation possible (9, 10). Moreover, we found that cytochrome c release, apoptosome formation, and caspase-3/7 activation could be observed upon induction of both apoptosis and another phenomenon, differentiation (11).

It seems that Apaf-1 is able to act as a tumour-suppressor gene (12). Intensive and diffusible cytoplasmic immunohistochemistry (IHC) staining has been reported locally for Apaf-1 molecules in normal skin, nevi and melanoma (13). It is proposed that Apaf-1 plays a role in the progression of the disease, as focal positivity was noticed in melanoma and also in less than 25% of all tumour cells from metastatic melanoma. Furthermore, an opposite correlation has been reported between Apaf-1 expression and the pathologic stage. Loss of heterozygosity at the Apaf-1 gene was compatible with decreased mRNA expression level in metastatic melanoma, weak disease occurrence and chemo-resistance (14).

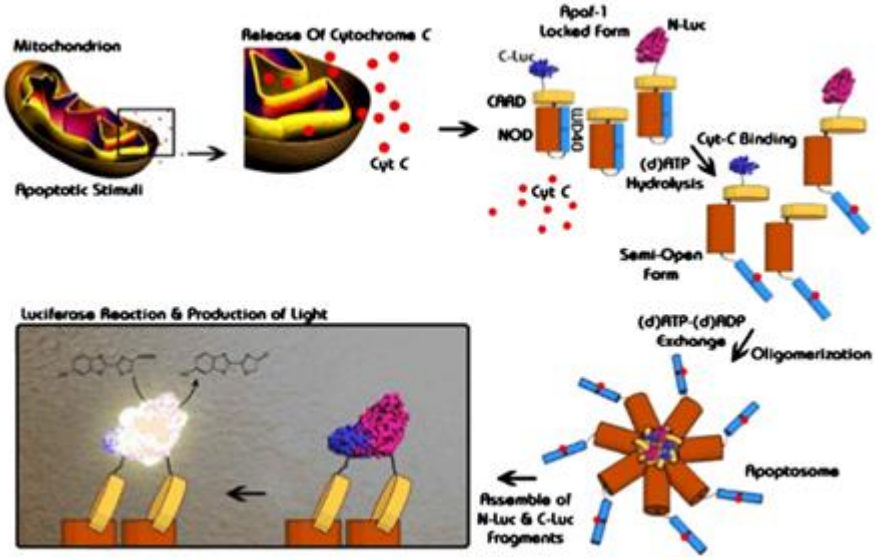

Figure 1. Apoptosis biosensor mechanism of action. Upon the induction of apoptosis, cytochrome *c* is released from the inter-membrane of mitochondria and causes the Apaf-1 molecule to switch from autoinhibited state into open form, through which apoptosome complex is formed. With the formation of apoptosome, fragments of luciferase are placed in the right position and emit a luminescent signal. Reprinted from (9) with permission from Biosensors and Bioelectronics

In colorectal cancer (CRC), an increase in the frequency of allelic disparity at the Apaf-1 locus has been correlated with tumour advancement (or enlargement) from adenoma to carcinoma to metastatic cancer (15). An exacerbated response to treatment has been signified in patients with rectal cancer enduring pre-operative radiotherapy with decreased Apaf-1 expression in the pre-treatment biopsy compared to those testing positive for the protein (16, 17). All the above studies relate to real cases of cancer or any disability related to changes in Apaf-1 expression or Apaf-1 function due to mutation(s) in its gene or promoter. However, in our three-year study, we have seen several unwanted mutations in the Apaf-1 gene, all of which were introduced during in-vitro studying. Since it seemed that the mutation rate of this gene was high, we decided to evaluate the factors affecting the production of these mutations during laboratory work. In this study we investigated several factors such as temperature, existence of repeated regions, and effects of different strains that might affect mutation rate of the gene. We report conclusions and suggest areas for future study, where mutation rate at repeated regions reaches the minimum.

Materials and Methods

Reagents

The following reagents were used in this study: restriction endonuclease DpnI (Fermentas), Ampicilin (Sigma), PrimeSTAR GXL DNA Polymerase (Takara Bio), the plasmid extraction kit (GeneAll Biotechnology), the gel purification kit and the polymerase chain reaction (PCR) purification kit (GeneAll Biotechnology).

Plasmid construct and mutation resource

In this study, we gather the data from unwanted

mutagenesis working with two primers which have that we used in our other study with the specific purpose of introducing a single-point mutation in 1558 nucleotide and changed the codon of Arg to stop the codon of UGA in the Apaf-1 gene. Since the other study has not yet been published, we will try to clarify and explain our PCR condition. The quick-change site-directed mutagenesis method (18) was used to introduce specific changes. As mentioned earlier, in addition to investigation of the mutation rate in the Apaf-1 gene, in the other study we were seeking to study the structure of a biosensor containing the Apaf-1 gene conjugated to split luciferase. Therefore, we used two different plasmids containing either N or C fragment of luciferase (Fig. 2). pcDNA3.1 vectors containing cDNA sequence encoding Nluc/ Apaf1 and Cluc/ Apaf1 were used as templates and Prime STAR GXL DNA Polymerase was used for amplification (Nluc and Cluc are two fragment of luciferase linked to Apaf-1; the other study represented an assay of luciferase complementation). Therefore, two oligonucleotide primers, each complementary to an opposite strand of pcDNA3.1 containing wild-type Apaf1 fused to luciferase fragments, were extended during temperature cycling by Prime STAR GXL DNA Polymerase, generating mutated plasmids containing staggered nicks. PCR conditions were: one cycle at 98ºC for 30 s, 20 cycles at 94ºC for 45 s, 60ºC for 45 s, 68ºC for 10 min, and finally 68°C for 10 min. Following temperature cycling, firstly the primary products were cleaned up using Gene All clean-up kit, and then the products were treated with Dpn I. The Dpn I endonuclease is specific for methylated and hemimethylated DNA, and is used to digest the parental DNA template and to select mutation-harbouring synthesized DNA. Thereafter, the nicked DNA vectors were transformed into DH5-α or XL10-Gold competent cells. A set of overlapping primers encoding the stop codon that were introduced into the cDNA contained in pcDNA3.1 have been listed in Table 1. The single mutants were prepared using primers 1 and 2, where nucleotides marked in bold indicate the mutated codons. Then, pcDNA3.1 vectors containing Nluc/ Apaf1 and Cluc/ Apaf1 were sequenced to confirm wanted and unwanted mutations (Macrogen).

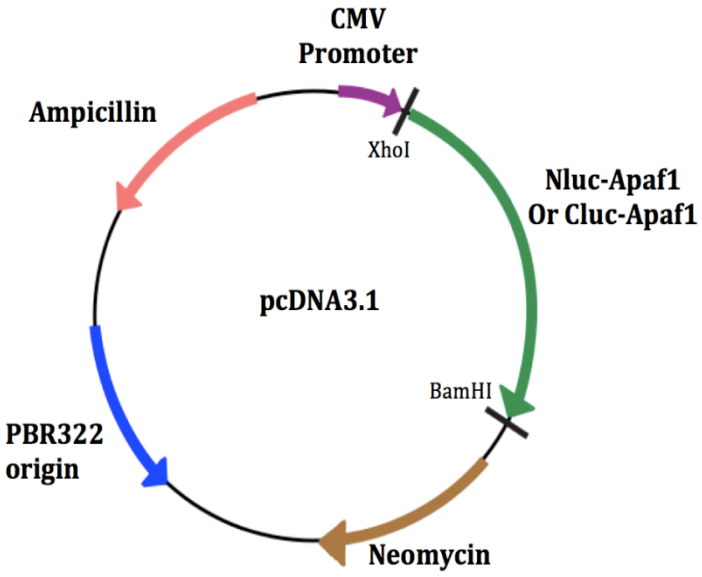

Figure 2. Scheme of pcDNA3.1 plasmid containing Apaf-1 gene linked to luciferase fragment. In this plasmid, the Apaf-1 gene attached to a luciferase fragment is flanked by Xho1 and BamH1, two restriction enzymes.

Table 1. The set of overlapping primers used in this study

Primer numbers	Primer names	Primer sequences
1	Forward	ATTTGTGGAATACTGACATATACTAGATGAA
2	Reverse	TTCATCTAGTATATGTCAGTATTCCACAAAT

Cell culture and transformation condition

Since the various transformation protocols with different reagents and in different time intervals may significantly affect the mutation rate, we here describe the applied method as follows.

The prepared competent cells were thawed from -70ºC onto wet ice and a number of 1.5 mL tubes pre chilled simultaneously. Upon thawing, 50 µL of competent cells were aliquoted into 1.5 mL tubes, into which 5 ng of plasmid DNA added and mixed gently by tapping the tube. After 30 min incubation on ice, the cells were heat-shocked at 42ºC for 45 seconds and reincubated on ice for 15 min. 950 µL of LB medium was then added to tube and shaken at 225 rpm at 37ºC for one hour to let the antibiotic resistance gene express. In this step a number of samples were also incubated at 18$^{°C}$ for five hours. The medium was then centrifuged and 900 ml of supernatant discarded; the subsequently deposited pellet was resuspended in the remaining 100 ml supernatant. Using a plate spreader, 100 µL of the reaction was spread onto LB medium under sterile conditions. When dried over 10 min, Petri plates were incubated overnight at 37ºC. Another plate was incubated at 18ºC for four days. Bacterial colonies were then collected and used as samples for colony PCR. Those positive colonies were selected for plasmid extraction and subsequently sequenced.

Finally, it should be mentioned that we used two different bacterial-competent cells, DH5α and XL10-Gold, in order to observe the possible influence of various strains on the obtained results.

Results

High rate of Apaf-1 gene recombination during genetic engineering. After PCR using the quick-change site-directed mutagenesis method and DpnI treatment, we transformed our PCR products into DH5-α. After 24 h incubation at 37ºC, each colony was transferred to LB medium in order to ensure a large amount of bacteria for plasmid extraction. Then, because the Apaf-1 gene with its conjugated luciferase fragment had been flanked with BamhI and XhoI restriction sites in pcDNA3.1 plasmid, each extracted plasmid was digested with these two restriction enzymes. As shown in Figure 3A, the double digestion of plasmids, which were chosen randomly from colonies, showed different patterns. In our study, due to self-recombination and high rate of mutation in the Apaf-1 gene several patterns with different sizes were observed, which in turn demonstrates how this gene has potential for a high rate of mutation. In Figure 3A from left to right, upon double digestion, instead of just two bands of plasmid and the gene of interest, different patterns with unreasonable sizes were observed in lanes

This observation indicates self-recombination and a high rate of mutation in the Apaf-1 gene. Lanes 4 and 5 appear as smears, which also reflects the previous hypothesis. Lane 12 is the DNA ladder. On the other hand, the single and double

digestion of plasmids extracted from the transformed colonies and the native one showed expected results, according to the pcDNA3.1 and Apaf-1 conjugated to either base pair number of the fragments of luciferase nucleotide (Fig. 3B). In Figure 3B, from left to right, lanes 2, 4, 7, 9 and 11 are single-digested and lanes 1, 3, 6, 8, and 10 are double-digested. Lane 5 is the DNA ladder.

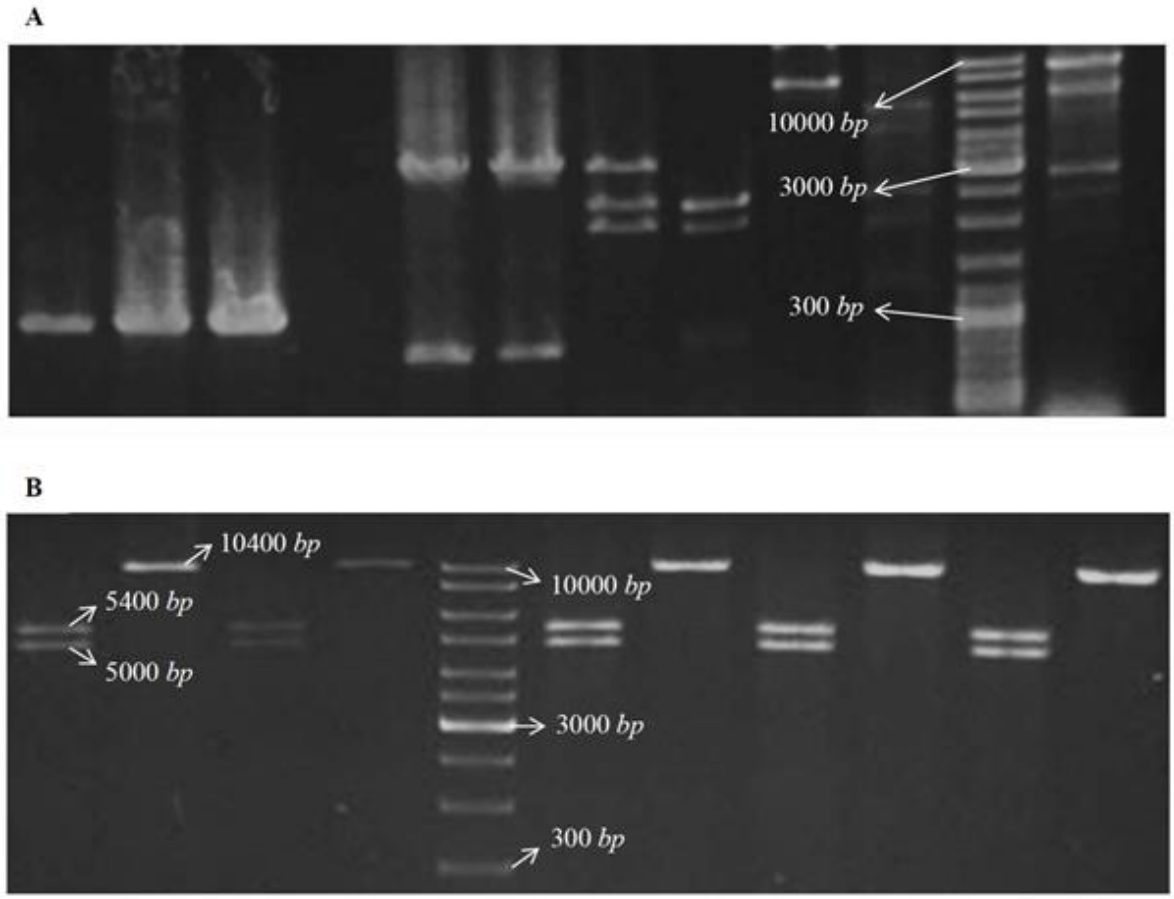

Figure 3. Digestion of different plasmids with BamHI and XhoI after incubation at two different conditions (A) Gel electrophoresis of 12 different pcDNA3.1 plasmids including Nluc-Apaf-1 after PCR using the quick-change site-directed mutagenesis method, transformation, plasmid extraction and double digestion. (B) Gel electrophoresis of five different pcDNA3.1 plasmids including Nluc/Apaf-1 (Apaf-1 and N-terminus luciferase fragment) just after transformation, plasmid extraction and single or double digestion. From left to right, lanes 1, 3, 6, 8, and 10 are digested with two restriction enzymes, lane 5 is the DNA ladder, and the others are digested with one restriction enzyme. Lanes 1 and 2 relate to the controls, which are not transformed. The 10400-base-pair band contains pcDNA3.1, Apaf-1, and N-terminus luciferase fragments. pcDNA3.1 is a 5400-base-pair vector and Nluc/Apaf-1 is a 5000-base-pair fragment.

Evaluation of mutation in two different temperatures and strains. In order to evaluate the probable role of temperature and bacterial strain in the rate of point mutation, an experiment was developed in which four groups were selected. The differences between these groups were related to their culture temperature and transformation bacterial strain. Two strains of *Escherichia coli***,** DH5-α and XL10-Gold, were used; half of the transformed bacteria were incubated at 18ºC and the other half at 37ºC. After transformation, we screened colonies by colony PCR with Apaf-1 forward and reverse

primers replicating the whole of the Apaf-1 gene. Among several colonies, only positive colonies in colony PCR were gathered, and after single and double digestion the non-recombinant plasmids were sequenced. Among 48 sequenced plasmids, 21 samples showed 38 point mutations; the others showed no mutation. As shown in Figure 4A, at 18ºC the mutation rate in DH5-α cells and XL10-Gold cells were very much alike; only, the DH5-α cells indicated a smoother rate of mutation more than the XL10-Gold cells. However, this difference increased at 37ºC so that the rate of mutation in the DH5-α cells was about 1.5 times higher than the XL10-Gold cells. The total number of mutations in the DH5-α cells was 1.6 times higher than the XL10-Gold cells (Fig. 4B).

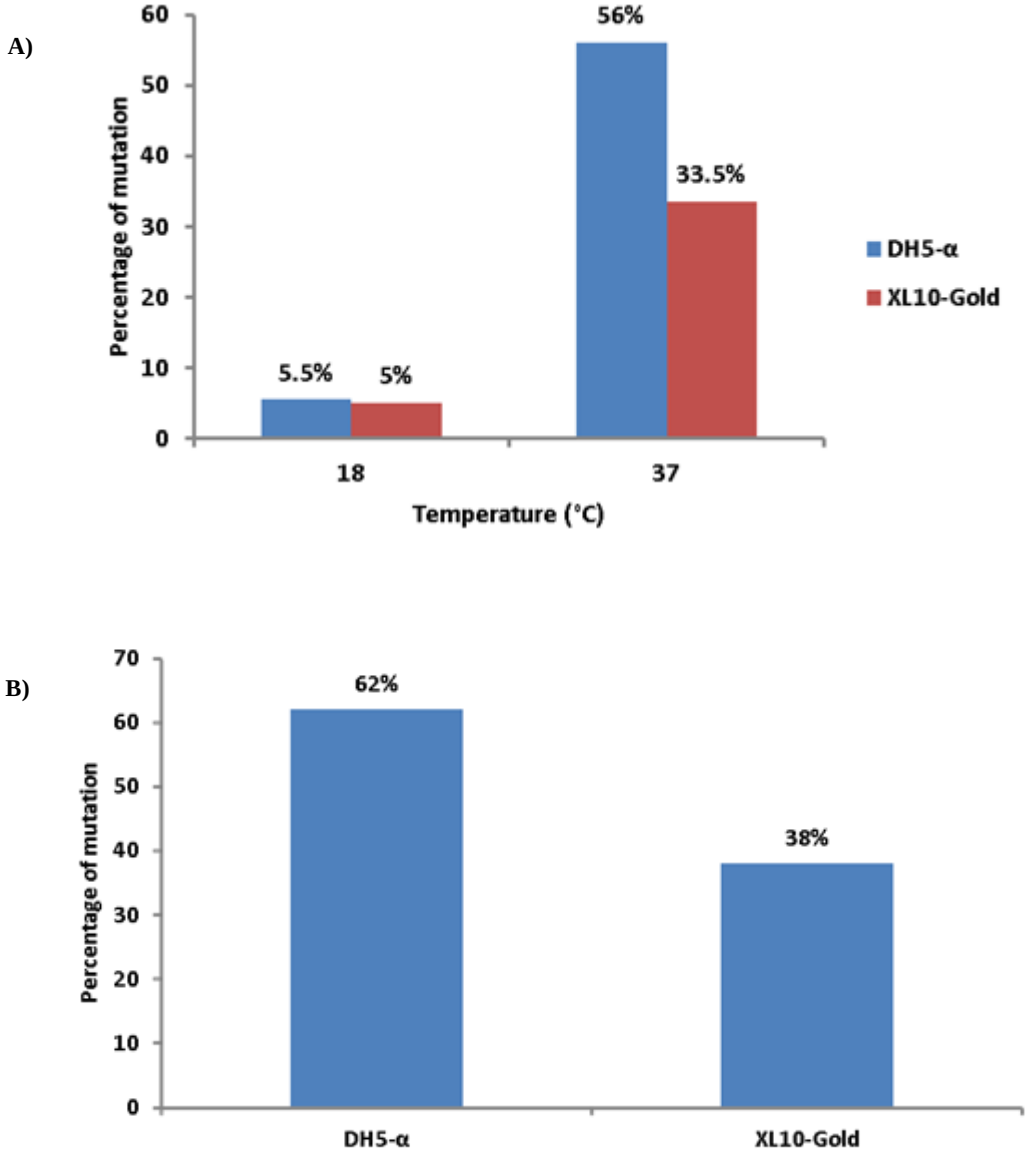

Figure 4. Relationship between two different strains and mutation rate. (A) Percentage of discovered mutations in DH5-α and XL10-Gold at two different temperatures, 18 and 37ºC, relative to the total mutations. (B) Overall percentage of mutations in DH5-α and XL10-Gold.

Evaluation of relationship between Apaf-1 domains and mutation rate. A large number of mutations, 21, was observed in the first WD-40 domain. As distance on both sides of this domain towards the carboxy and ammonia terminus of the Apaf-1 gene increased, the number of mutations decreased (Fig. 5A). The second WD-40 domain of Apaf-1 also indicated 12 distinct mutations. Both WD-40 domains showed several repeated regions. However, NOD, which did not have any noticeable repeated regions, showed five mutations.

Different kinds of mutations and probable hot spots for mutations in the Apaf-1 gene. As shown in Figure 5B, there was significant variation in the number of different mutations. Most of the mutations, 22, were classified under the base-substitution type. It should be noted that for all the base-insertion, -deletion, and -substitution mutations that were observed, just one nucleotide changed. Nevertheless, the number of nucleotides that changed in duplication mutations was surprising: 37. In order to find hot spots particularly susceptible to mutations, we consider all mutations. In spite of the fact that mutation locations were dispersed across three domains– CARD, first and second WD40 repeats – we found only two probable hot spots for spontaneous mutation (Table 2). One of these sequences indicated duplication of 37 nucleotides with two base-substitution mutations; the other indicated three separate base-substitution mutations in three different plasmids.

Table 2. Two probable hot spots susceptible to spontaneous mutation in the Apaf-1 gene. Wild-type and mutant-sequence alignments were performed using EMBL-EBI multiple-sequence alignment server. As indicated, a 37-nucleotide fragment was duplicated and base substitution was seen in the same fragment. In base insertion, three nucleotides were inserted in different sites of the gene. (*) Represents complete identity and (-) represents the absence of specific nucleotides

Duplication and Base substitution	Wild-type Mutant	TGTGATGTTTTCTCCTGATGGATCATCATTTTTGACATCTTCTGATGACCAGACAATCAG TGTGATGTTTTCTCCTGATGGATCATCATTTTTGACATCTTCTGATGACCAGACAATCAG **********
	Wild-type Mutant	GCTCTGG----------GAGACAAAGAAAGTAT GCTCTGGGAGACAAAGAAAGTATGACCAGACAATCAGGCTCTGAGAGACAAAGAAAGTAT ******* **********
	Wild-type Mutant	GTAAGAACTCTGCTGTAATGTTAAAGCAAGAAGTAGATGTTGTGTTTCAAGAAAATG GTAAGAACTCTGCTGTAATGTTAAAGCAAGAAGTAGATGTTGTGTTTCAAGAAAATG **********
Base insertion	Wild-type Mutant	TACCAAGTTTTCATCTACCTCTGCTGACAAGACTGCAAAGAT-CTGG-AGTTTTGATCTC TACCAAGTTTTCATCTACCTCTGCTGACAAGACTGCAAAGATTCTGGAAGTTTTGATCTC ********** **** **********
	Wild-type Mutant	CTTTTGCCACTTCATG-AATTGAGGGGCCACAACGGCTGTGTGCGCTGCTCTGCCTTCTC CTTTTGCCACTTCATGGAATTGAGGGGCCACAACGGCTGTGTGCGCTGCTCTGCCTTCTC ********** **********
	Wild-type Mutant	TGTGGACAGTACCCTGCTGGCAA TGTGGACAGTACCCTGCTGGCAA **********

Discussion

Apoptosis is a phenomenon that causes disposal of unwanted cells from multicellular organisms, in which more than 200 proteins play roles. Genetic studies have revealed that all of these proteins are under delicate regulation. One of the most important of these regulations is related to pre-mRNA splicing (19). Alternative splicing has a pivotal role in producing four Apaf-1 variants that differ in two regions. One of them is NH2-terminal 11-amino acid, inserted between the caspase recruitment domain and the ATPase domains; the other is the 43-amino acid COOH-terminal insert between the fifth and sixth WD40 repeat regions. According to functional analysis, the COOH-terminal WD-40 repeat insertion is very

important and Apaf-1 isoforms without this insertion at WD-40 repeats cannot activate procaspase-9 in response to releasing cytochrome c, and are abundant in some tumour cell lines (20). But what is the probable relationship between our abundant observed recombination and this splicing? Pre-mRNA processing is guided by specific nucleotide sequences which attract specific factors – U1/2 snRNP, U2AF, hnRNP, to name but a few – in order to catalyse pre-mRNA processing and, in some kinds of pre-mRNA processing, catalysis of intron removal by sequences (21). If these sequences exist in pre-mRNA of Apaf-1, it will be logical to conclude that these also exist in the Apaf-1 gene and may lead to unwanted self-recombination, as we saw in our study (Fig. 3). Furthermore, we did not see any recombination in native Apaf-1 just after transformation and plasmid extraction (Fig. 3B) as we observed recombination in Apaf-1 after PCR using the quick-change site-directed mutagenesis method and transformation (Fig. 3A). It therefore seems that this self-recombination needs some propelling factors, such as a nick, created after PCR using the quick-change site-directed mutagenesis method in a plasmid.

Although studies have already focused in detail on the expression of the Apaf-1 gene (17), Apaf-1 spontaneous mutations have not been considered sufficiently yet. Frameshift mutations in Fas, Apaf-1, and Bcl-10 in gastro-intestinal cancer of the microsatellite mutator phenotype have been reported and all Apaf-1 mutations have been related to WD-40 repeats (22). Some studies have also reported direct relation between different kind of mutations in Apaf-1 and tumour progression, as well as mutations at coding repeat sequences such as in mismatch repair-deficient human cancers (23, 24). Here, we evaluated mutations in the Apaf-1 gene in laboratory work, not in real case studies. In our study we examined the essential factors, bacterial strains and temperatures. As shown in Figure 4B, the total percentage of mutations in DH5-α was higher than XL10-Gold. This difference at 37ºC was significant; at 18ºC the difference was partial (Fig. 4A). Generally speaking, the higher the temperature in a reaction, the greater the motion: this principle in chemical reactions is comparable with mutation rate, due to its chemical nature. Despite the logically different mutation rates at these two temperatures, the presumed reason for the different number of mutations between DH5-α and XL10-Gold strains seems more complicated to explain. It may simply be related to different recombination- and repair-dependent enzyme activity.

As indicated in Figure 5A, it seems that there are two factors which affect mutation rate in Apaf-1. First and foremost, repeat regions, especially the first WD-40 domain, are particularly susceptible to mutation. The second factor is the distance between a specific location and WD-40 repeats; in particular the role of the first WD-40 domain is more significant than that of the second one. In this regard, as the distance between a location and the first WD-40 domain increases, the mutation rate of this sequence will decrease. These results could be due to the spatial specific conformation of WD-40 domains, which affects their flanked sequences.

According to our observations, most mutations were widespread through WD-40 repeats and neighbour sequences, rather than concentrated on a few specific hot-spot sequences. We found only two probable hot-spot sequences. One of these sequences had a surprising 37 nucleotide base pairs, and was

not only susceptible to duplication but also to base-substitution mutation (Table 2). The length of this sequence is significant, and it may be for this reason that we see a lower frequency for this mutation compared with others (Fig. 5B). Additionally, we observed a significant difference in mutation rate between different kinds of mutation. A full explanation of this will require further study at molecular level.

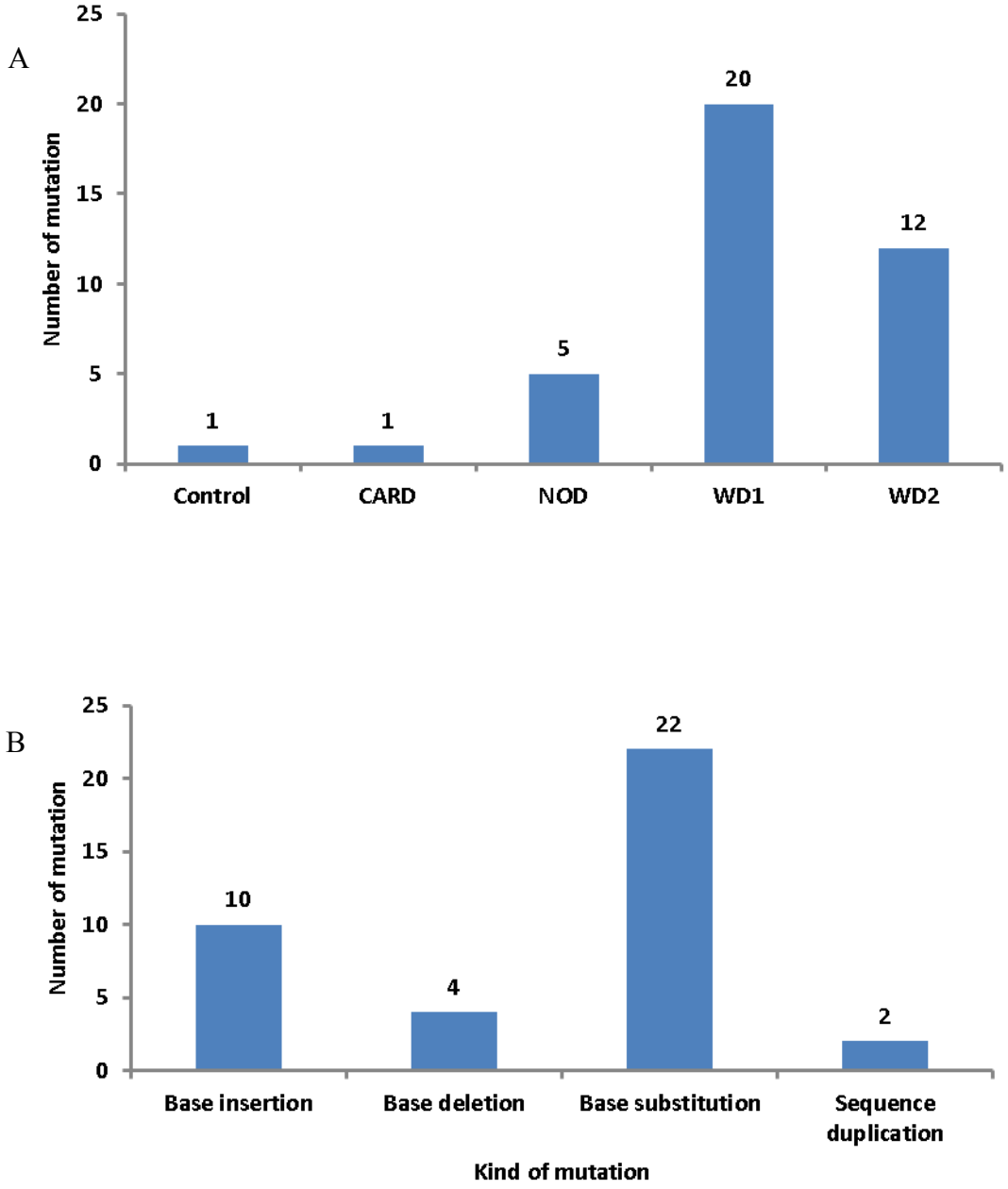

Figure 5. Number of mutations in each domain of Apaf-1 and classification of different types of mutation in Apaf-1 gene (A) Number of discovered mutations in each domain of Apaf-1 protein, composed of four subdomains: CARD, NOD, WD1 and WD2. Control is the number of discovered mutations in 48 plasmids without repeated regions, which were sequenced in our previous research by Macrogen. As indicated, the greater number of mutations are in WD repeat regions. (B) Number of each type of mutation observed in the Apaf-1 gene after PCR and transformation.

As calculated in the experimental approaches, the occurrence of spontaneous mutations is similar within the genome of a wide variety of organisms, but significantly different between groups. In RNA viruses with the calculated 104-base genome, the mutation rates are almost one per genome per replication for lytic viruses and almost 0.1 per genome per replication for retroviruses and retrotransposon. In contrast, in microbial chromosomal DNA the mutation rates are nearly 1/300 per genome per replication. Thus, there is a huge inverse relationship between the rate per base pair and the genome size varying from 6×10^3 to 4×10^7 bases or base pairs. In higher eukaryotes, mutation rates are roughly 0.1–100 per genome per sexual generation, but at the time indecipherable from 1/300 per cell division per effective genome (fraction of genome with no neutral mutations). Some of the driving forces that develop various mutation rates may now be specified (25, 26). However, the rate of spontaneous mutations in our gene of interest is higher than that reported in usual genes; we suggest this is due to point-mutation induced by WD-40 repeats. In molecular biology, this is a process by which mutations are accumulated in DNA. Genomic evidence indicates that repeat-induced point mutation occurs or has occurred in a variety of organisms (27).

To sum up, we reported here for the first time the spontaneous mutations and recombination in the Apaf-1 gene during in-vitro laboratory study. These results can pave the way for reducing unwanted spontaneous mutations and recombination by paying attention to the choice of more appropriate strains and appropriate incubation conditions, such as optimum temperature. Moreover, the finding of two hot-spot sequences which are susceptible to spontaneous mutation and several widespread mutations upon WD-40 repeats and neighbour sequences will be invaluable in the search for more accurate information between cancer and mutation in these sequences.

REFERENCES

1. Fulda, S. and Debatin, K. (2006) Extrinsic versus intrinsic apoptosis pathways in anticancer chemotherapy. *Oncogene*, 25, 4798-4811.

2. Green, D.R. and Reed, J.C. (1998) Mitochondria and apoptosis. *Science*, 281, 1309-1311.

3. Riedl, S.J. and Salvesen, G.S. (2007) The apoptosome: signalling platform of cell death. *Nature* , 8, 405-413.

4. Danial, N.N. and Korsmeyer, S.J. (2004) Cell death: critical control points. *Cell*, 116, 205-219.

5. Riedl, S.J., Li, W., Chao, Y., Schwarzenbacher, R. and Shi, Y. (2005) Structure of the apoptotic protease-activating factor 1 bound to ADP. *Nature*, 434, 926-933.

6. Reubold, T.F., Wohlgemuth, S. and Eschenburg, S. (2011) Crystal structure of full-length Apaf-1: how the death signal is relayed in the mitochondrial pathway of apoptosis. *Structure*, 19, 1074-1083.

7. Adrain, C., Slee, E.A., Harte, M.T. and Martin, S.J. (1999) Regulation of apoptotic protease activating factor-1 oligomerization and apoptosis by the WD-40 repeat region. *J. Biol. Chem.*, 274, 20855-20860.

8. Srinivasula, S.M., Ahmad, M., Fernandes-Alnemri, T. and Alnemri, E.S. (1998) Autoactivation of procaspase-9 by Apaf-1-mediated oligomerization. *Mol. Cell*, 1, 949-957.

9. Torkzadeh-Mahani, M., Ataei, F., Nikkhah, M. and Hosseinkhani, S. (2012) Design and development of a whole-cell luminescent biosensor for detection of early-stage of apoptosis. *Biosens. Bioelectron.*, 38, 362-368.

10. Azad, T., Tashakor, A. and Hosseinkhani, S. (2014) Split-luciferase complementary assay: applications, recent developments, and future perspectives. *Anal.Bioanal. Chem.*, 406, 5541-5560.

11. Akbari-Birgani, S., Hosseinkhani, S., Mollamohamadi, S. and Baharvand, H. (2014) Delay in Apoptosome Formation Attenuates Apoptosis in mouse Embryonic Stem Cell Differentiation. *J. Biol. Chem*, M113. 536730.

12. Campioni, M., Santini, D., Tonini, G., Murace, R., Dragonetti, E., Spugnini, E.P. and Baldi, A. (2005) Role of Apaf-1, a key regulator of apoptosis, in melanoma progression and chemoresistance. *Exper. Dermatol.*, 14, 811-818.

13. Mustika, R., Budiyanto, A., Nishigori, C., Ichihashi, M. and Ueda, M. (2005) Decreased expression of Apaf-1 with progression of melanoma. *Pig. Cell Res.*, 18, 59-62.

14. Fujimoto, A., Takeuchi, H., Taback, B., Hsueh, E.C., Elashoff, D., Morton, D.L. and Hoon, D.S. (2004) Allelic imbalance of 12q22–23 associated with APAF-1 locus correlates with poor disease outcome in cutaneous melanoma. *Cancer Res.*, 64, 2245-2250.

15. Umetani, N., Fujimoto, A., Takeuchi, H., Shinozaki, M., Bilchik, A.J. and Hoon, D.S. (2004) Allelic imbalance of APAF-1 locus at 12q23 is related to progression of colorectal carcinoma. *Oncogene*, 23, 8292-8300.

16. Zlobec, I., Vuong, T. and Compton, C.C. (2006) The predictive value of apoptosis protease-activating factor 1 in rectal tumors treated with preoperative, high-dose-rate brachytherapy. *Cancer*, 106, 284-286.

17. Zlobec, I., Minoo, P., Baker, K., Haegert, D., Khetani, K., Tornillo, L., Terracciano, L., Jass, J.R. and Lugli, A. (2007) Loss of APAF-1 expression is associated with tumour progression and adverse prognosis in colorectal cancer. *Eur. J. Cancer*, 43, 1101-1107.

18. WANG, W. and Malcolm, B. (1999) Two-stage PCR protocol allowing introduction of multiple mutations, deletions and insertions using QuikChange Site-Directed Mutagenesis. *Biotechniques*, 26, 680-682.

19. Jiang, Z.-H. and Wu, J.Y. (1999) Alternative splicing and programmed cell death. *Experimental Biol. Med.*, 220, 64-72.

20. Benedict, M.A., Hu, Y., Inohara, N. and Núñez, G. (2000) Expression and Functional Analysis of Apaf-1 Isoforms EXTRA WD-40 REPEAT IS REQUIRED FOR CYTOCHROME cBINDING AND REGULATED ACTIVATION OF PROCASPASE-9. *J. Biol. Chem.*, 275, 8461-8468.

21. Schwerk, C. and Schulze-Osthoff, K. (2005) Regulation of apoptosis by alternative pre-mRNA splicing. *Mol. Cell*, 19, 1-13.

22. Yamamoto, H., Gil, J., Schwartz Jr, S. and Perucho, M. (2000) Frameshift mutations in Fas, Apaf-1, and Bcl-10 in gastro-intestinal cancer of the microsatellite mutator phenotype. *Cell Death Differ.*, 7, 238-239.

23. Duval, A. and Hamelin, R. (2002) Mutations at Coding Repeat Sequences in Mismatch Repair-deficient Human Cancers Toward a New Concept of Target Genes for Instability. *Cancer Res.*, 62, 2447-2454.

24. Ionov, Y., Yamamoto, H., Krajewski, S., Reed, J.C. and Perucho, M. (2000) Mutational inactivation of the proapoptotic gene BAX confers selective advantage during tumor clonal evolution. *P. Natl. Acad. Sci.*, 97, 10872-10877.

25. Drake, J.W., Charlesworth, B., Charlesworth, D. and Crow, J.F. (1998) Rates of spontaneous mutation. *Genetics*, 148, 1667-1686.

26. Nachman, M.W. and Crowell, S.L. (2000) Estimate of the mutation rate per nucleotide in humans. *Genetics*, 156, 297-304.

27. John Clutterbuck, A. (2011) Genomic evidence of repeat-induced point mutation (RIP) in filamentous ascomycetes. *Fungal Genet. Biol*, 48, 306-326.