Journal of the Egyptian Society of Haematology & Research, Vol. 11, No. 1, March: 9-15, 2015

Role of RAD51G135C SNP in the Risk of Developing AML and its Impact on Survival

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ABSTRACT

Background: RAD51 (Rec A homolog of E.coli) is a polymorphic gene and one of the central proteins in homologous recombination-DNA-double-strand breaks (HR-DNA-DSB) repair pathway, which is vital in maintaining genetic stability within a cell.

Objectives: To determine the relation of RAD51 SNPs (single nucleotide polymorphism) with the risk susceptibility to and impact on the survival of adult AML.

Patients and Methodos: The study included 60 de Novo adult AML patients and 60 age and sex matched healthy unrelated Egyptian control subjects. RAD51G135C was tested by PCR-RFLP. Response to induction chemotherapy was evaluated. Patients were followed-up for Overall Survival (OS).

Results: RAD51G135Cgenotypes distribution in AML cases (wild 75%, variant 25%) was not significantly different from the control group (wild 81.7%, variant 18.3%) (p=0.375). However, the median survival for patients with mutant RAD51 gene was significantly lower than that for patients with wild RAD51 (25 days versus 4.5 months respectively, p 0.005).

Conclusion: The presence of SNP in RAD51has no impact on the risk of developing adult de novo AML but has poor prognostic impact with significant poor survival of the AML patients.

Key Words: *AML – SNP – RAD51 – PCR-RFLP.*

INTRODUCTION

The mechanisms for de novo AML genesis are still rarely understood. Evidence suggests that radiation, smoking, obesity and exposure to chemical carcinogens are considered as its possible risk factors. Nevertheless, AML only develops in a small proportion of people exposed to these environmental and lifestyle risk factors, indicating that the host genetic background might play a critical role in its genesis **[1]**.

DNA damage repair and cell-cycle checkpoints are the most important defense mechanisms against mutagenic exposures. The most important DNA-repair pathways in human cells are: Direct repair, Base Excision Repair (BER), Nucleotide Excision Repair (NER), Mismatch Repair (MMR) and double strand break repair (DSB repair). DSB is divided into Homologous Recombination (HR) for example RAD51 and non-homologous end joining (NHEJ) and Translesion DNA Synthesis (TLS). Each pathway repairs a different type of lesion **[2]**.

Polymorphisms in DNA repair genes, including those involved in base excision repair, nucleotide excision repair, mismatch repair and double strand break repair have been implicated in carcinogenesis. Common polymorphisms in DNA repair genes may alter protein function and an individual's capacity to repair damaged DNA. Deficits in repair capacity may lead to genetic instability and tumor genesis **[3]**.

RAD51 is a central protein in the HR repair pathway, binding to DNA and promoting ATP-dependent homologous pairing and strand transfer reactions. The most important polymorphism identified for RAD51 is G135C SNP in 5' untranslated region. The RAD51 G135C polymorphism is associated with RAD51 protein overexpression **[4]**. RAD51 interacts with and is stabilized by XRCC3, during strand invasion and cross-strand resolution. RAD52 may modulate these activities through its RAD51-interacting region. The ability of RAD52 to induce homologous recombination requires its binding to the p34 subunit of Replication Protein A (RPA); which is a DNA-binding protein that

plays an essential role at replication centers as well as stalled replication forks in the HR repair system **[5]**.

FLT3 (Fms-Like Tyrosine Kinase 3) is activated in about 30% of AML cases. Internal Tandem Duplication (ITD) mutations in FLT3 are associated with the risk of relapse in AML **[6]**. Some patients with AML have dual mutations of ITD and the tyrosine kinase domain. The dual mutations induce resistance to FLT3 inhibitors and chemotherapeutic agents. The mechanism underlying the resistance was shown to be mediated by STAT5 activation, leading to upregulation of Bcl-x (L) and RAD51. Another study has shown that the FLT3 inhibitor PKC412 and the silencing of FLT3 by RNA interference repress RAD51 in cells with FLT3-ITD mutations but not in cells with intact FLT3. These data suggest that RAD51-mediated HR activity contributes to resistance to therapy in AML with FLT3-ITD mutations **[7]**.

Accumulating evidence suggests that concurrent radiotherapy with Epidermal Growth Factor Receptor (EGFR) inhibitors provides a survival benefit in a variety of cancers, such as those of the lung, head, and neck. The EGFR inhibitor Erlotinib was shown to inhibit radiation-dependent activation of RAD51, indicating that repressed RAD51 contributes to the effect of the concurrent therapy **[8]**.

Thus, some tyrosine kinase inhibitors may not only inhibit growth-promoting signals but also overcome resistance to chemotherapy and radiotherapy by down regulating the HR pathways mediated by RAD51 and its associated proteins **[6]**.

PATIENTS AND METHODS

Patients: The study was performed on 60 de Novo AML patients presented to the Medical Oncology Department, NCI, Cairo University in the period from June 2012 to January 2014. Sixty age and sex matched apparently healthy unrelated individuals selected from blood donors served as a control group; they included 58 (58%) males and 42 (42%) females with an age range of 17 to 59 and a median of 32.5 years. The study was approved by the IRB of the NCI, Cairo University and an informed consent was obtained from each subject before enrollment.

Methods: All patients were subjected to complete history taking and clinical examination as well as radiological investigations as indicated. The diagnosis of AML was done according to standard methods (WHO, 2008) and classification was made using the French-American-British (FAB) criteria **[9]**.

Laboratory investigations included:

- Complete and differential blood count.
- Serum chemistry including liver function and kidney function tests.
- Bone marrow aspiration and examination of Romanowsky stained smears, supplemented with cytochemical stains such as Peroxidase (MPO) or Sudan Black Stain (S.B.B), Estrases, Acid Phosphatase and PAS when indicated.
- Immunophenotyping using monoclonal antibodies and flow cytometric analysis (REF).
- Conventional karyotyping was performed for all cases (REF).
- FISH as a complementary tool to conventional cytogenetics when indicated (REF).

RAD51 G135C genotyping:

Blood or bone marrow samples were obtained into EDTA tubes. DNA was extracted from WBCs by salting out method (REF) followed by Polymerase Chain Reaction (PCR) as described by Voso et al., **[10]**. Amplification of the extracted DNA was performed in 25µl reaction mix containing 200ng DNA, 200ng each primer, 1.5mmol/L $MgCl_2$, and 1 unit Taq DNA polymerase in a total volume of 25µL. Following initial denaturation at 95ºC for 7 minutes, 40 PCR cycles were done. Amplification conditions included initial denaturation at 95ºC for 5 minutes followed by 30 cycles of 94ºC for 1 minute, 62ºC for 1minute, and 72ºC for 1 minute with a final elongation step at 72ºC for 7 minutes. The primer sequences were:

- RAD51F (5′-TGGGAACTGCAACTCATCTGG-3′) and
- RAD51R (5′-GCGCTCCTCTCTCCAGGCAG-3′).

Enzymatic digestion of the PCR products was performed using one unit MvaI restriction enzyme. Digestion was performed at 37º for 30 minutes in 20µl reaction mix containing 10ul fast digest restriction enzyme mixture (7ul H_2O + 2ul buffer + 1u enzyme) + 10ul PCR product.

The PCR and the digestion products were visualized with ethidium bromide after electrophoresis on 2% agarose gel at 100 volts for 30min.

Wild type RAD51 (GG) showed 86 and 71bp fragments of complete digestion, the homozygous RAD51 (CC) retained the 157bp product of the amplification step and the heterozygous RAD51 (GC) showed the three bands Fig. (1).

Patients were followed-up for a period of 20 days to 72 months with a median of 5 months; Overall survival (OS) and disease free survival were analyzed in context of RAD51 G135C genotypes.

Treatment plan:

Patients received standard induction chemotherapy using cytosine arabinoside and anthracycline as 7 and 3 protocol. Patients who achieved CR were consolidated by the same regimen then HLA typing was done for those below 40 years of age with good general condition ,–ve inv (16) and –ve *t* (8,21). Those with identical donor were referred for allergenic BMT. Those with no HLA identical donor, those with favorable risk (+ve inv 16 or *t* (8,21)) and those 40 years were given 4 cycles of HAM consolidation. Intrathecal prophylaxis was given only for cases with AML M5 (high risk of CNS disease) after achieving CR by induction chemotherapy. Triple intrathecal prophylaxis was given every 8 weeks for a total of 6 injection using methotrexate 15mg, Ara-C 40mg and dexamethasone 4mg.

For patients with AML M3 induction treatment with All Trans-Retinoic Acid (ATRA) (45m/m^2 orally daily in 2 divided doses until CR or for a maximum of 3 months) and adriamycin (30mg/m^2 for 3 days for 3 courses). patients who achieved complete remission received maintenance treatment of ATRA (45mg/m^2 orally daily for 2 weeks every 3 month), methotrexate (20mg/m^2 IV once weekly) and 6 mercaptopurine (60mg/m^2 daily) for 2 years.

Response to induction chemotherapy:

Complete remission was defined as a normocellular BM containing less than 5% blasts and showing evidence of normal maturation of other marrow elements, no circulating blast cells, no evidence of extramedullary leukemia and recovery of granulocytes to 1500/µl and platelets to 100,000/µl. Unfavorable outcome included refractory cases (didn't achieve CR) and early death (death within 30 days of diagnosis and before evaluation of the response).

Statistical analysis:

Data was analyzed using IBM SPSS advanced statistics version 20 (SPSS Inc., Chicago, IL). Numerical data were expressed as mean and standard deviation or median and range as appropriate. Qualitative data were expressed as frequency and percentage. Chi-square test was used to examine the relation between qualitative variables. For quantitative data, comparison between two groups was done using either Student *t*-test or Mann-Whitney test (non-parametric *t*-test) as apprporiate. Comparison between 3 groups was done using Kruskal-Wallis test (non-parametric ANOVA). Survival analysis was done using Kaplan-Meier method and comparison between two survival curves was done using log-rank test. Odds Ratio (OR) with it 95% Confidence Interval (CI) were used for risk estimation. All tests were two-tailed. A *p*-value <0.05 was considered significant.

Overall survival was measured from the date of diagnosis to death or last follows-up.

RESULTS

This study included 60 de novo AML patients, 28 males (46.7%) and 32 females (53.3%) with an age range of 18-78 years with a median age of 32 years.

The presenting total leucocytic count in the study cases ranged from 2.2 to 183X10^9/L with mean of 60.7±56.5 and a median of 43.5X10^9/L. The platelet count ranged from 6 to 297X10^9/L with a mean of 59.4±54.7 and a median of 40 X10/L. Hemoglobin ranged from 4.2 to 12gm/dl, with a mean of 7.69±1.8 and a median of 7.3 gm/dl. Blasts in peripheral blood ranged from 3 to 90% with a mean and SD of 40.4%±28 and a median of 32%. The mean percentage of blasts in marrow was 70.1%±21.4, the median was 77% and the range was 22-97%. All the studied cases were classified according to FAB classification. The most frequent was M2 followed by M4 while the least was M5 and M7; no M6 cases were encountered in our cohort. Cytogentics and molecular genetics findings showed that the majority of our patients (70%) had normal karyotype. The details of patient's characteristics are shown in (Tables 1,2).

RAD51 G135C genotypes in AML vs. control:

The wild type RAD51 (135 GG) was encountered in 45 (75%) AML patients compared to 49 (81.7%) controls, the heterozygous genotype RAD51 (135 GC) in 14 (23.3%) AML patients compared to 11 (18.3%) controls and the homozygous genotype RAD51 (135 CC) in 1 (1.7%) AML patients compared to 0 (0%) controls. The frequency of both polymorphisms (GC + CC) (mutant type) versus the wild type (GG) was found to be 25% versus 75% in the AML group and 18.3% versus 81.7% in the control group with (*p*.value=0.375).

Association of RAG51 G135C genotypes with various clinical and hematological parameters:

There was no association between RAD51 genotypes on one side and age (*p*=0.065), gender (*p*=0.999), Hb (*p*=0.322), TLC (*p*=0.785), platelets (*p*=0.188), PB blasts (*p*=0.713), BM blasts (*p*=0.745) or cytogenetics (*p*=0.329) on the other side. For FAB subtypes, we had only 2 cases for M5 and M7. So no association could be done statistically for all subtypes.

Response rate:

Complete Remission (CR) was achieved in 24/60 patients (40%). Twenty one out of 45 patients with wild RAD51 genotype showed CR (46.7%), while only 3/15 patients with the variant form achieved CR (20%). Although there is a trend towards better response achievement among patients with wild RAD51, this difference was not statistically significant (*p*=0.068). The single patient with homozygous RAD51 genotype failed to achieve CR.

Overall Survival:

The median follow-up period for survival of the entire group was 2 month (range: 0.233-24). The median survival for the whole group was 3 month (95% CI 1.463-4.537) with cumulative survival at 6 month of 33.2% Fig. (2). The cumulative survival at 6 months for patients with RAD51 wild genotype was 38% with median survival of 4.5 month (95%CI: 1.739-7.261) versus only 20% cumulative survival at 6 month and median of 25 days (95% CI: 0.518-1.149) for those with RAD51 mutant disease (*p* 0.005, Fig. (3)). Early death occurred in 13 (21.7%).

Table (1): Characteristics of 60 adult acute myeloid leukemia patients.

Parameter	Findings
Age in years: Median (range)	32 (18-78)
Gender: No. (%)	
Male	28 (46.7)
female	32 (53.3)
TLC X 10^9/L: Median (range)	43.5 (2.2-183)
Hb gm/dL: Median (range)	7.3 (4.2-12)
PLT X 10^9/L; median (range)	40 (6-297)
PBB %, median (range)	32 (3-90)
BMB %, median (range)	77 (22-97)

TLC : Total Leukocyte Count.
PLT : Platelet Count.
PBB : Peripheral Blood Blasts.
BMB: Bone Marrow Blasts.
CR : Complete Remission.
OS : Overall Survival.

Table (2): FAB subtypes and karyotypes of 60 adult acute myeloid leukemia patients.

FAB types	No.	(%)	Cytogenetic	No.	(%)
M1	12	(20)	Normal	42	(70)
M2	25	(41.7)	Inv 16	4	(6.7)
M3	6	(10)	*t* (8;21)	6	(10)
M4	13	(21.7)	*t* (15;17)	6	(10)
M5	2	(1.3)	hyperploidy	1	(1.7)
M7	2	(1.3)	trisomy 8	1	(1.7)

Table (3): Summary of ORs for various contrasts of RAD51G135C polymorphisms among acute myeloid leukemia patients (Li et al., 2014) **[14]**.

Genetic contrast models	Ethnicity	OR (95% CI)	*p*-value
GC vs. GG	Caucasian	1.38 (0.61-3.09)	0.441
	Asian	1.04 (0.81-1.34)	0.762
	Other	1.27 (0.91-1.79)	0.161
	Total	1.22 (0.83-1.79)	0.303
CC vs. GG	Caucasian	1.07 (0.39 3)	0.891
	Asian	1.27 (0.61-2.61)	0.523
	Other	1.24 (0.38-4.08)	0.727
	Total	1.2 (0.71-2.05)	0.494
CC vs. GG/GC	Caucasian	1.02 (0.36-2.86)	0.972
	Asian	1.25 (0.61-2.57)	0.622
	Other	1.19 (0.36-3.93)	0.772
	Total	1.17 (0.69-2)	0.558
CC/GC vs. GG	Caucasian	1.35 (0.61-2.96)	0.457
	Asian	1.06 (0.83-1.36)	0.659
	Other	1.27 (0.92-1.77)	0.153
	Total	1.22 (0.84-1.77)	0.299

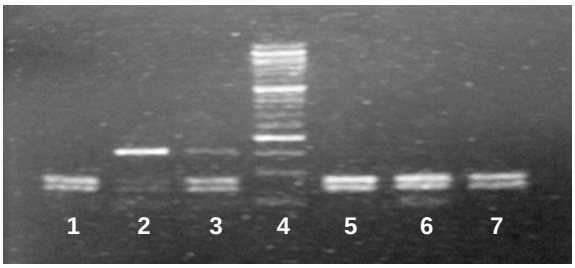

Fig. (1): PCR products after digestion for RD51G135C using MvaI restriction enzyme.

Lanes 1, 5, 6, 7: RD51G135C wild type (86bp, 71bp).
Lane 2: RD51G135C Homozygous (157bp).
Lane 3: RD51G135C heterozygous variant (157bp, 86bp, 71bp).
Lane 4: 50bp ladder.

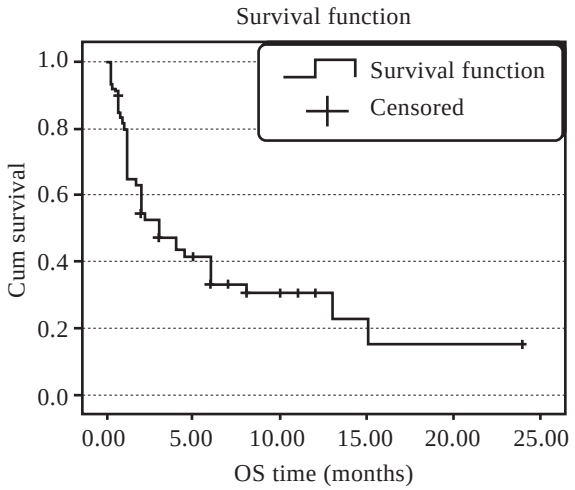

Fig. (2): Overall survival of 60 AML patients.

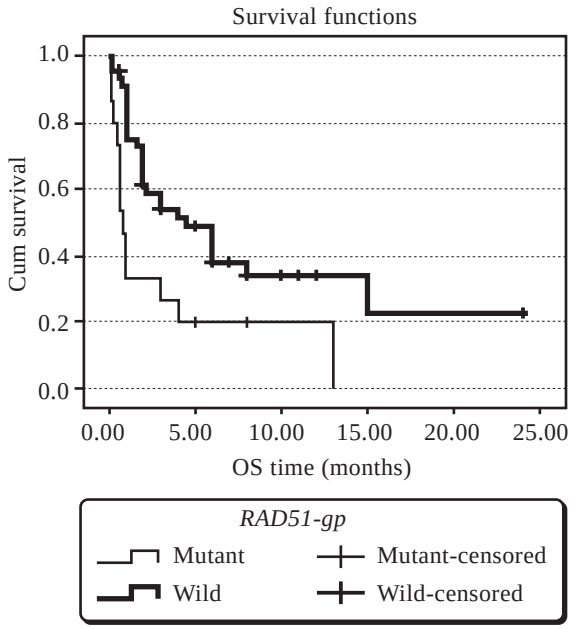

Fig. (3): Overall survival according to RAD51 status in 60 AML cases (*p* 0.005).

DISCUSSION

The polymorphisms of RAD51 which belongs to homologous recombination, part of double strand break repair, was analyzed. Polymorphisms that occur in DNA repair genes affect DNA repair capacity. Common genetic variations in genes involved in DNA repair or response to genotoxic stress may influence both cancer susceptibility and treatment outcome **[11]**.

Some studies supported this hypothesis and showed that RAD51 polymorphism is associated with significant increase in the risk of development of AML **[3,12,13]** in contrast to the results of the current study. However, this association between RAD51 genotypes and development of AML could not be confirmed in 2 Meta analyses. A systematic search of three databases including Pub. Med. and EMBASE for the period up to 20 February 2013 which identified 43 relevant studies was performed by Li et al., **[14]**. Six eligible studies were eventually selected for RAD51 (1764 cases and 3469 controls). Pooled Odds Ratios (ORs) and 95% Confidence Intervals (CIs) for the risk of AML associated with RAD51 were appropriately calculated based on fixed or random effects models. The quality of studies was evaluated using the Newcastle-Ottawa Scale (NOS). Subgroup analyses were performed among Asian, Caucasian and other populations. The pooled results showed that the leukemia risk was not significantly associated with RAD51 and the same results were obtained among any subgroup analysis. This meta-analysis provides evidence that the RAD51 polymorphisms are not associated with an increased risk of AML in the total population as shown in (Table 3).

Also Cheng et al., **[15]** believed that the results of previous studies on the association between RAD51 G135C polymorphism and cancer risk have been inconclusive, partially because of the relatively small sample size of most studies. Therefore, they performed a meta-analysis including 6836 cases and 8507 controls from 22 case-control studies to evaluate the association between RAD51 G135C polymorphism and risk of acute leukemia, Squamous Cell Carcinoma of the Head and Neck (SCCHN), colorectal cancer and ovarian cancer. The overall population analysis showed no significant association between RAD51 G135C polymorphism

and risk of acute leukemia, SCCHN, colorectal cancer or ovarian cancer in any genetic model. However subgroup analysis showed that individuals with GG genotype are more likely to develop SCCHN than other genotypes.

In the present study there was a trend towards better achieving CR among patients with wild RAD51 genotype although not statistically significant (p=0.068). However patients with wild RAD51 genotype had significant better OS versus patients with mutant genotype (p 0.005). In a study by Liu et al., **[16]** RAD51 (G135C) genotypes were analyzed in 372 Chinese patients with AML by PCR-RFLP or PCR. The Complete Remission (CR) rate, adverse effects, Overall Survival (OS), and Relapse-Free Survival (RFS) were compared among the groups with different genotypes. They concluded that RAD51 gene polymorphism was significantly related to response to therapy, adverse effects, and prognosis of AML with better outcome in patients with wild RAD51 genotype.

Also, Bănescu et al., **[17]** reported that the RAD51 gene polymorphism showed significant unfavorable outcome among AML patients.

This was explained by Miyagawa **[6]** who concluded that RAD51-mediated Homologous Recombination (HR) activity contributes to resistance to therapy in AML patients.

On the contrary, Bhatla et al., **[18]** concluded that RAD51 gene polymorphism did not influence the outcome of AML therapy in the study of de novo AML patients.

Statistical comparison between RAD51 genotypes as regards treatment outcome revealed no statistically significant difference between different genotypes in an Egyptian study of 50 de novo AML patients. Thus, RAD51 gene polymorphism was found to have a non-significant impact on the risk of treatment failure which might be explained by the small sample size. Yet, the percentage of patients with unfavorable outcome (relapse and death) among patients expressing polymorphic RAD51 G135C allele (78% and 100% for heterozygous [G/C] and homozygous [C/C] types. respectively) was higher than that of the patients with favorable outcome (remission) **[13]**.

Also another study of 103 de novo AML patients with positive inv (16)/t (16;16) (CBF-beta-MYH1) that were followed-up and retrospectively analyzed found that RAD51 G135C polymorphism has no significant impact on the prognosis among this group of patients **[19]**.

In conclusion, the presence of SNP in RAD51 has no impact on the risk of developing adult de novo AML. However our results showed that RAD51 polymorphism has poor prognostic impact with significant poor survival among patients with mutant variant. Also there was trend towards better response to induction chemotherapy for patients with GG genotypes versus those with CC and GC genotypes.

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