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t(12;21) and t(9;22) Fusion Gene Transcript Level at Diagnosis: Correlation to Hematological and Clinical Parameters

EMAN R. RADWAN, M.D.*; EMAN O. RASEKH, M.D.** and ALAA EL-HADDAD, M.D.***

The Department of Clinical & Chemical Pathology, Faculty of Medicine, Clinical Pathology Department** and Pediatric Oncology***, NCI, Cairo University*

ABSTRACT

Background: Acute lymphoblastic leukemia (ALL) is the most common pediatric malignancy. Recurrent chromosomal translocations with fusion gene transcript product are present in a subset of patients with defined prognostic impact. However, the level of the fusion gene transcript at diagnosis is extremely variable between individual cases.

Objectives: The aim of this study was to evaluate the fusion gene transcript level at diagnosis of two chimeric genes, one with good and one with bad prognostic implication.

Patients and Methods: The study was performed on 40 newly diagnosed pediatric ALL patients, 21 with t(12;21) and 19 with t(9;22). Fusion gene transcript level was evaluated by quantitative real time PCR. Positive cell lines were used as the 100% level and patient's values calculated as a ratio to the cell line; the median value was used to discriminate between high and low levels. Data were correlated to other hematological and clinical parameters and impact on overall survival was studied.

Results: The fusion gene transcript levels were extremely variable in both groups. There was a trend for a higher level associated with t(9;22) (p=0.08). Positive correlation with Hemoglobin (Hb) level and DNA index was encountered in the t (9;22) patients (r=0.511, p=0.025 and r=0.513, p=0.035 respectively). The transcript level at diagnosis had no impact on overall survival. No difference in any of the hematological or clinical parameters was encountered between patients with low and high transcript level.

Conclusions: Fusin gene transcript level at diagnosis is extremely variable between individual patients; however this variability was not reflected on treatment outcome. It was not associated with other prognostic or biological features except for positive correlation of *BCR/ABL* transcript level with Hb level and DNA Index. Quantitative evaluation of fusion gene transcript is simple, reliable and highly sensitive making it an excellent tool for potential MRD detection.

Key Words: *ALL – t(12;21) – t(9;22) – Fusion gene transcript.*

INTRODUCTION

Acute lymphoblastic leukemia is the most common hematological malignancy in children **[1]**.

Marked progress has been achieved in unraveling the biological nature of the disease allowing its stratification into various subsets, each with a specific therapeutic approach **[2-6]**. This was associated with marked improvement in treatment outcome with ~90% cure rate **[2,6]**. Nevertheless, a minority of cases shows initial resistance to therapy and some cases do relapse after initial good response **[7-9]**. Among the known prognostic parameters tumor burden exemplified mainly by total leucocyte count is well documented. Another prognostic factor is the type of chimeric fusion gene detected in some cases with t(12;21) associated with the best and t(9;22) with the worst outcome **[10]** The level of chimeric fusion gene transcript at diagnosis may also reflect the tumor burden. The transcript level at diagnosis show marked variability between different cases but tend to be constant at the individual case level allowing its use for detection of minimal residual disease **[11,12]** which has been widely applied **[13-16]**. However the impact of the quantity of transcribed fusion product at diagnosis, its relation

to other prognostic parameters and potential impact on outcome was not addressed.

In this study, we performed true quantitation of the fusion genes t(12;21) and t(9;22) in 40 newly diagnosed pediatric ALL patients. We aimed to verify if the fusion gene transcript level, as an indicator of tumor burden, would correlate to other prognostic parameters and be reflected on disease outcome.

PATIENTS AND METHODS

The study was performed on 40 pediatric ALL patients harboring either t(12;21) or t(9;22). They included 21 males and 19 females with an age range of 1.7-17.5, a mean of 8.7±5.3 and a median of 7.0 years.

All patients presented to the outpatient of the Pediatric Oncology Department, NCI, Cairo University. The study was performed according to Helsinki declaration for studies on human subjects. The protocol was approved by the NCI Institutional Review Board and a written informed consent was obtained from all patients parents/guardians.

Cases were diagnosed according to standard methods including complete blood count (CBC), bone marrow (BM) aspirate, cytochemistry as indicated, immunophenotyping and testing for the common fusion genes. According to the latter 21 cases with t(12;21) and 19 with t(9;22) were included in the study.

Detection of chimeric fusion gene transcripts:

Cell lines having each of the chimeric fusion genes were used, namely:

- K562 for t(9;22) p210.
- A synthetic RNA for t(9;22) p190.
- REH for t(12;21).

HL60: Negative cell line.

RNA was extracted using Quiagen RNeasy extraction (QIA amp RNA blood Mini kit, Catalog no. 52304) according to manufacturer's instructions. RT was performed using high capacity cDNA synthesis kit (Applied Biosystems).

Real-time PCR using ABI 7700 (Taqman, BI-Applied Biosystems) was used to test for the presence of chimeric fusion genes resulting from the t(9; 22) (q34; q11), t(12; 21) (p13; q22).

Real-time RT-PCR by ABI 7700-Taqman:

A total volume of 50µl/reaction was used in Taqman plates. Ten µl cDNA (100ng), 25µl 2X TaqMan Universal Master Mix (Roche P/N 04914058001) and the volume of the primers and probes and DEPC treated water varied according to each translocation. The working concentration for the primers was 900nM for *BCR/ABL* p190 and 300nM for *TEL/AML1*, *BCR/ABL* p210. The final concentration of the probes was 100nM. Cycling conditions were 50 °C for 2min, 95°C for 10min then 40 cycles of amplification with denaturation at 95°C for 15sec, annealing, and extension at 60°C for 1min.

Cell spiking at various levels provide a range of controls at relevant analytic and clinical decision points. Cell spiking was performed by creating a matrix of positive and negative control cell lines. This matrix was later extracted and used to verify analytical measurement range for the quantitative assays.

For diagnostic tests, both positive and negative control samples were included.

Positive controls included:

- RNA extracted from positive cell lines.
- Amplification control (*GAPDH*).
- Spiked controls: RNA extracted from HL60 which has been spiked with positive controls from the fusion of interest. The spiking RNA concentrations should match those as noted above and should include [CML t(9;22), ALL t(9;22), or t(12;21) in HL60] in spiked ratios of 1:10, 1:100, 1:1,000, 1:10,000, and 1:100,000. The OP1 Spike set stops at 1:10,000.

Negative controls included:

- RNA from a negative individual or cell line HL60.
- A no template control (NTC) or water blank.

Sequences of primers and probes are presented in Table (1).

Table (1): Fusion genes primer and probe sequences.

Fusion gene	Primer sequence
t(9;22) *BCR-ABL1* (P210)	
B3A2 Forward	5' GCT GAC CAT CAA TAA GGA AGA TGA
Reverse	3' GAT GCT ACT GGC CGC TGA
B2A2 Forward	5' TGT GTG AAA CTC CAG ACT GTC CA
Reverse	3' TGG CCG CTG AAG GGC
t(9;22) *BCR-ABL1* ALL/ P190	
Forward	5' CGC AAG ACC GGG CAG A
Reverse	3' GCT CAA AGT CAG ATG CTA CTG GC
t(12;21) *ETV6/RUNX1*	
Forward: *TEL* Exon 5:	5' CCC CGC CTG AAG AGC AC
TEL Exon 3:	5' TCC ACC CTG GAA ACT CTA TAC ACA
Reverse *AML1* Exon 2	3' CGT CTC TAG AAG GAT TCA TTC CAA GTA
AML1 Exon 3:	3' TGA AGC GGC GGC TCG T
AML1 Exon 4:	3' GAG GAA GTT GGG GCT GTC
Probe	**Sequence**
t(9;22) *BCR-ABL1* CML/(p210)	
B3A2 Probe	FAM-ATG GGT TTC TGA ATG TCA TCG TCC ACT CA-TAMRA
B2A2-Probe	FAM-AGC ATT CCG CTG ACC ATC AAT AAG GAA GA-TAMRA
t(9;22) *BCR-ABL1* ALL/(p190)	FAM-CAA CGA TGA CGA GGG CGC CTT C TAMRA
t(12;21) *ETV6/RUNX1*	
p1	FAM-CCA TGC CCA TTG GGA GAA TAG CA-TAMRA
p2	FAM-ACA GCC GGA GGT CAT ACT GCA TCA GAA-TAMRA

• TaqMan Probes for CML t(9;22) *BCR/ABL1* (B3A2 and B2A2), ALL p190 *BCR/ABL1*, t(12;21) *TEL-AML1* (*ETV6/RUNX1*) fusion genes were manufactured by PE Applied Biosystems Probes. Probes come as 100µM concentrations. The final concentration in the reaction is 100nM, except for B3A2 and B2A2 probes which have a 50nM each final concentration in the reaction. They were diluted 1:40 with molecular grade water just prior to use.

Result reporting and interpretation:

1- For diagnostic and MRD samples, the presence of product is indicated by the appearance of signal above the critical threshold (Ct) for the experiment.
2- Standard curves were then generated using the dilutions of the K562 for t(9;22) p210, synthetic RNA for t(9;22) p190 and REH for t(12;21) positive controls.
3- The input of positive target (K562, synthetic RNA, and REH) and internal target (GAPDH) for the spiked controls and patient samples were calculated from the standard curves.
4- A normalized ratio of t(9;22) CML, t(9;22) ALL and t(12;21) input/control input was calculated.
5- The calculated ratio was then compared to the spiked controls to determine the amount of target present in the patient sample.

The cell line is considered as 100% positive. The value at diagnosis was divided by the value of the cell line and expressed as folds.

Statistical methods:

SPSS version 18.0 was used for data management. For quantitative data mean ± standard deviation described it, with median and range when appropriate. Log values for fold expression of fusion gene were used for its correlation with different quantitative variables using Pearson correlation analysis or Spearman Rho test when subgroup analysis was done. Non parametric *t*-test compared means of 2 independent groups and Chi-square/Fisher exact compared proportions of independent groups. Kaplan Meier estimated survival and log ranks compared curves. *p*-value was considered significant at ≤0.05 level.

RESULTS

The study was performed on 40 newly diagnosed pediatric precursor B-ALL patients positive for the fusion genes t(12;21), t(9;22), including 21 males and 19 females with an age range of 1.7-17.5, a mean of 8.7±5.3 and a median of 7.0 years. They included 21 cases with t(12;21) and 19 with t(9;22).

Characteristics of patient are presented in Table (2). Patients with t(9;22) had significantly higher age (p=0.021) and TLC (p=0.03) and significantly lower CR (p=0.005).

As regards immunophenotyping, pre-B was the dominant phenotype in all groups comprising 14 (66.67%) cases in t(12;21) and 12 (63.16%) in t(9;22). The other phenotype in the remaining

cases was c-ALL comprising 7 (33.33%) in t(12;21) and 7 (36.48%) in t(9;22). No pro-B phenotype was encountered in our cohort. Aberrant CD 13 and CD33 expression was encountered each in one case (4.76%) with t(12;21). Expression of both CD13 and CD33 was found in 3 cases (14.29%) with t(12;21) and one case (5.3%) with t(9;22).

Table (2): Characteristics of patients with t(12;21) and t(9;22).

Parameter	t (12;21) (21 cases)	t(9;22) (19 cases)	*p*-value
Age (years)	4.37±1.79 4 (1.7-7)*	12.34±5.61 14 (9-17)	0.021
TLC x 10^9/L	30.47±46.51 12.6 (1.7-182)	135.38±202.91 51.6 (0.9-850)	0.03
Hb g/dl	7.38±2.52 8 (2.9-13.1)	7.452±1.963 7 (4-12.3)	0.98
Platelets x 10^9/L	66.58±126.92 35 (2-605)	72.18±84.29 46 (5-296)	0.61
BM blast%	87±12.52 93 (55-98)	79.47±21.11 85 (55-98)	0.18
Splenomegaly	12 (57.1%)**	11 (57.9%)	0.96
Hepatomegaly	13 (61.9%)	10 (52.6%)	0.55
Lymph nodes	6 (28.6%)	5 (26.3%)	0.87
CNS	1 (4.8%)	3 (15.8%)	0.33
CR	19 (100%)	10 (62.5%)	0.005

* Mean ± SD, median (range). ** No (%).
TLC : Total leucocytic count.
Hb : Hemoglobin.
BM : Bone marrow.
CNS : Central nervous system involvement.
CR : Complete remission.

As regards DNA index, only two patients (9.5%) with t(12;21) and one patient (5.3%) with t(9;22) fell in the good prognostic group with a DNA index of ≥1.16 – <1.6. The majority of cases, 11 (52.4%) with t(12;21) and 13 (68.4%) with t(9;22), fell in the hyperdiploid range ≥1 <1.16. Hypodiploidy with DNA index <1 was encountered in 8 (38.1%) with t(12;21) and 5 (26.3%) with t(9;22).

The fusion gene transcript level at diagnosis was extremely variable; for t(12;21) it ranged from 0.00049 to 91.1 with a mean of 10.743± 20.153, and a median of 3.89. For t(9;22), the variability was more pronounced; it ranged from 0.0004 t0 522.03 with a mean of 44.59±122.84 and a median of 0.32. There was a trend of higher level in the t(9;22) group but it did not attain statistical significance (p=0.08). The fusion gene transcrip level for individual cases are presented in Table (3).

Table (3): Fusion gene transcript level at diagnosis for Pediatric acute lymphoblastic leukemia cases with t(12;21) and t(9;22).

Patient No.	t(12;21)	t(9;22)
1	1.75	0.16
2	91.11	0.0004
3	33.9	0.04
4	10.89	0.22
5	21.46	0.14
6	2.79	0.002
7	15.39	0.02
8	5.75	0.17
9	6.88	0.86
10	0.0014	0.46
11	3.23	0.17
12	2.78	57.4
13	0.00049	168.31
14	1.05	522.03
15	4.91	68.14
16	3.89	25.06
17	4.406	0.32
18	1.843	1.62
19	0.74	2.215
20	10	
21	2.84	

Correlations of fusion transcript level at diagnosis for t(12;21) and t(9;22) are presented in Table (4). t(12;21) fusion transcript level at diagnosis showed a trend of negative correlation with TLC (r=–0.378, p=0.09) and a poor statistically insignificant positive correlation with BM blast% (r=0.272, p=0.233). t(9;22) fusion transcript level at diagnosis showed a statistically significant positive fair correlation with both Hb level (r=0.511, p=0.025, Fig. 1A) and DNA index (r=0.513, p=0.035, Fig. 1B).

Table (4): Correlation between transcript level at diagnosis and other prognostic parameters.

Parameter	t(12;21): No 21		t(9;22): No 19	
	r	*p*-value	*r*	*p*-value
Age	–0.011	0.962	–0.075	0.761
Hemoglobin	–0.157	0.496	0.511	0.025
Total Leukocyte Count	–0.378	0.091	0.000	0.999
Platelets	0.214	0.351	–0.144	0.558
Bone Marrow blasts %	0.272	0.233	0.127	0.606
DNA index	–0.057	0.853	0.513	0.035

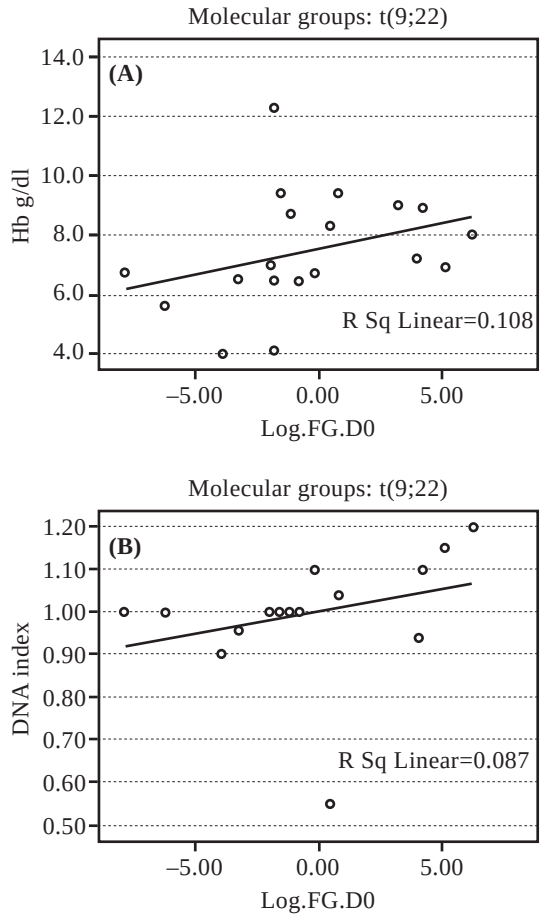

Fig. (1): Correlation between t(9;22) transcript level at diagnosis and (A) Hb level (B) DNA index.

The median fusion gene transcript level at diagnosis was used as the discriminating limit between high and low levels. For t(12;21) the median was 3.98; 11 (52.3%) patients had ≤3.98 and 10 (47.6%) patients had >3.98. For t(9;22) the median transcript level at diagnosis was 0.32; 10 (52.6%) patients had ≤0.32 and 9 (47.3%) patients had >0.32. By comparison between patients with high and low transcript levels at diagnosis in both groups, all prognostic parameters; including age, gender, organomegaly, lymphadenopathy and CNS involvement were comparable with no statistically significant differences between both categories.

The follow-up period varied from one week to 30 months with a median of 7 months. For t(12;21) patients with low transcript level showed mean survival time of 24.08±3.49 months and a median of 13.73 months versus 23.15±3.58 and 17.99 months in patients with high transcript level Fig. (2). The difference was not statistically significant (p=0.71).

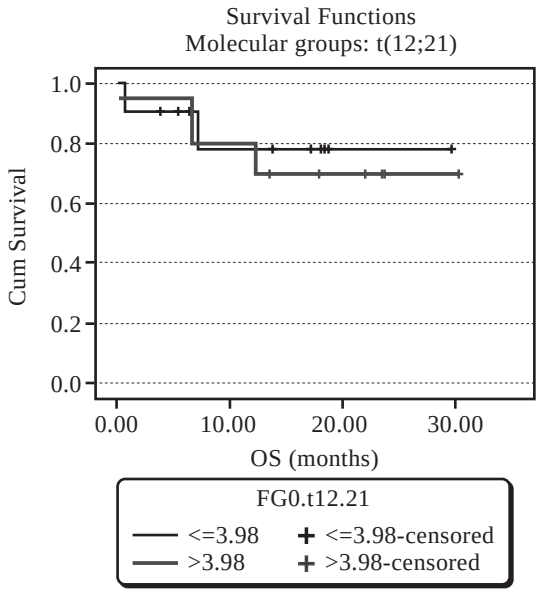

Fig. (2): Overall survival (OS) for t(12;21) patients with high and low transcript levels at diagnosis (p=0.71).

For t(9;22) the low transcript level patients had a mean survival time of 10.58±2.78 and a median of 7.16 months versus 6.96±2.9 and a median of 2.84 months for patients with high transcript level (Fig 3). The difference in survival was not statistically significant (p=0.48).

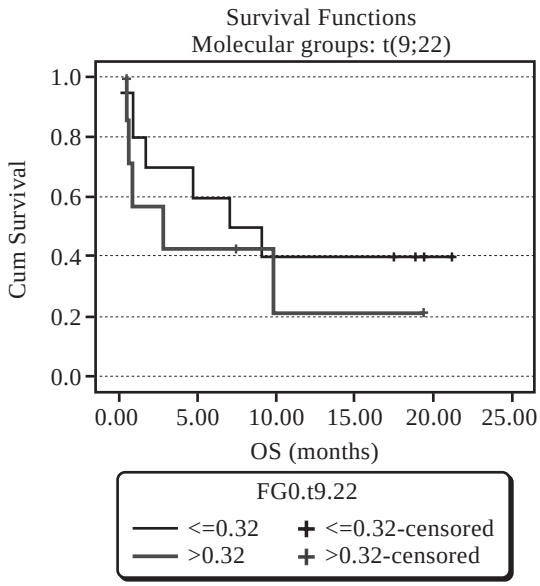

Fig. (3): Overall survival (OS) for t(9;22) patients with high and low transcript levels at diagnosis (p=0.48).

On comparing the survival of group I t(12;21) and group II t(9;22), t(12;21) patients showed mean survival time of 23.82±2.54 and a median

of 17.19, while t(9;22) patients showed mean survival time of 9.58±2.15 and a median of 7.16 months (Fig. 4). The difference was statistically significant (p=0.0114).

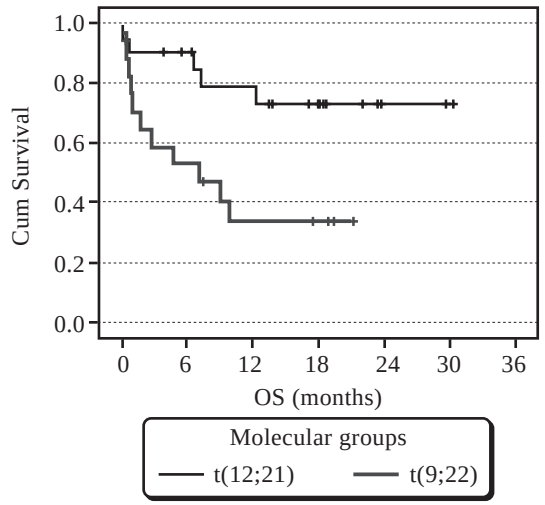

Fig. (4): Overall survival of t(12;21) and t(9;22) patients (p=0.0114).

DISCUSSION

In this work, we quantitatively estimated the transcript level of the fusion gene at diagnosis in 40 newly diagnosed pediatric ALL cases, 21 with the good prognostic t(12;21) and 19 with the bad prognostic t(9;22) translocation. We aimed to verify, if the fusion transcript level as an indicator of tumor burden would correlate with other prognostic parameters or impact OS.

We compared patient's characteristics between the t(12;21) and the t(9;22) positive groups.

In concordance with previous reports, age and TLC were significantly higher in patients with t(9;22) (p=0.021 and 0.03 respectively) **[18-20]** while CR was significantly higher in patients with t(12;21) (p=0.005) **[20,21]**.

In our study pre-B was the dominant phenotype in both groups comprising (66.67%) in t(12,21), 63.16% in t(9;22). The other phenotype in the remaining cases was c-ALL, no pro-B phenotype was encountered. This is in agreement with previous studies reporting that pre-B is the dominant phenotype in *BCR-ABL* positive adult and childhood ALLs **[20,22-25]**. But it was claimed that CD10+ cALL is the dominant phenotype in t(12;21) **[26]**.

By comparison between different groups regarding the DNA index only one case with t(12;21) and one case with t(9;22) lied in the good prognostic group ≥1.16 – <1.6. This is comparable to a previous study by Borkhardt et al., **[27]** who reported DNA index of ≥1.16-<1.6 in only 2/59 cases with t(12;21) and >1.6 in two more cases. A slightly higher number was reported by Arico et al., **[28]** where 5/37 cases were hyperdiploid ≥1.16 – < 1.6. However, the numbers in all the studies are too few to calculate actual frequency.

In the current study, the fusion gene transcript level at diagnosis showed marked variability. This is in agreement with other studies; Pallisgaard et al., **[29]** reported that the transcript levels of *TEL-AML1* at diagnosis may vary up to 14 fold after normalization to β2–microglobulin. Drunat et al., **[12]** detected variability in t(12;21) level at diagnosis by measuring relative copy number (*TEL-AML1*: TBP(TATA box-binding protein) ratio in 21 positive samples as determined by RQ-PCR; it ranged from 0.15-5.8. Marked variability in fusion gene transcript level at diagnosis was previously reported **[17,30-32]**; this was overcome by normalization to the house keeping gene used in the study allowing the compensation for the variation in RNA quality and day to day variation but they did not mention a range.

In the current study, there was a trend for a higher fusion gene transcript level at diagnosis in the t(9;22) compared to t(12;21) group (p=0.08). This comparison was not addressed in other studies **[17,30,31]**. Taking in account, the worse prognosis of the t(9;22), a higher transcript level might indicate a more aggressive behavior.

In our study there was an insignificant poor positive correlation between transcript level of t(12;21) at diagnosis and BM blasts % (r=0.272, p=0.233) and an insignificant poor negative correlation and TLC (r=–0.378, p=0.09).

In the current study a statistically significant positive fair correlation was encountered between transcript level of t(9;22) at diagnosis and Hb level (r=0.511, p=0.025). High Hb level at diagnosis signifies the rapid development of the malignancy; this association might signify that the high transcript level is also reflecting the aggressiveness and rapid progression of the

BCR-ABL positive ALL. There was also statistically significant positive fair correlation between transcript level of t(9;22) at diagnosis and DNA index (r=0.513, p=0.035). DNA index reflects the amount of DNA per cell which might explain the increase in the fusion gene transcript level.

In our study we chose the median value of the transcript level at diagnosis to discriminate between high and low transcript levels. Both groups were compared for the prognostic parameters including gender, lymphadenopathy, hepatomegaly, splenomegaly, CNS involvement and development of CR. There was no statistically significant difference between patients with high and low transcript levels within either the t(12;21) or t(9;22) groups; this goes in line with previous studies **[21,31,33]**.

By comparing the survival of patients with high and low transcript levels at diagnosis for t(12;21) or t(9;22), no statistically significant difference was encountered; this is in agreement with others **[21,31,33]**. However by comparison of the overall survival of patients with t(12;21) and t(9;22), the difference was statistically significant (p=0.0114); this is in concordance with previous reports **[8,9,34,35]**.

In the current study, there was no relapses among patients with t(12;21) included in our study and this is in concordance with previous studies **[12,35]**. However some studies showed late relapses among t(12;21) positive patients at low frequency **[33,36,37]**; this difference may be attributed to sample size, follow-up period and different treatment protocols i.e. (BFM-ALL) 95, (EORTC) 58 881; our patients received total XV. All contemporary protocols now include high dose L-asparginase which might explain some conflicting results reported from earlier trials **[38]**.

In conclusion, fusion gene transcript level at diagnosis showed marked variability between different patients for both t(12;21) and t(9;22); however this variability was not reflected on treatment outcome. The fusion gene transcript level at diagnosis was not associated with other prognostic or biological features except for positive correlation of *BCR/ABL* transcript level with Hb level and DNA Index. Quantitative evaluation of fusion gene transcript is simple, reliable and highly sensitive making it an excellent tool for follow-up by MRD detection.

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