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Evaluation of Cytoplamic Nucleophosmin1 and P27 Expressions in Acute Myeloid Leukemia and Their Relationship with Clinicopatholological Findings

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Abstract

Objectives: Nucleophosmin (NPM) is a nucleolar phosphoprotein which plays role in ribosome biogenesis and centrosome duplication. Changes in NPM expression has been reported in various malignancies. In 2008 WHO classification for acute myeloid leukemias (AML), a provisional subgroup harboring NPM mutation, which leads to its cytoplasmic localization, has been introduced. P27 is a CDK inhibitor which is disabled in various malignancies. Cytoplasmic NPM1 (cNPM1) and P27 expression in AML cases and their relation with survival and other clinical/laboratory findings, have been investigated in this study. **Methods:** Thirty, histologically confirmed, AML cases that had underwent bone marrow biopsy (BMB) between 2010 to 2013 in Quaem university hospital, participated the study. cNPM1 and P27 expressions were evaluated using IHC staining on BMB samples. The clinical and laboratory as well as survival data were extracted from hospital data or through contact with patients. **Results:** cNPM1 and P27 expression rates were 29% and 23%, respectively. There was a significant relation between cNPM1 expression as well as P27 expression and high (>10000/µl) WBC count (P=0.010 and P= 0.033, respectively). There were no significant differences between survival of patients with cNPM1 or P27 expression and the other patients. **Conclusions:** cNPM1 and P27 expressions may be associated with higher WBC count in AML. Their expressions were not associated with survival in this study. Further studies with larger sample size as well as concurrent investigation of other genetic abnormalities are needed to clarify the exact prognostic impact of these markers in AML.

Key words: Nucleophosmin, NPM1, cytoplasmic NPM1, P27, AML, Survival.

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Introduction

Acute myeloid leukemia (AML) is a heterogeneous group of disorders with different clinical behavior and outcome. The traditional morphological classification system has changed dramatically based on progression of knowledge about recurrent genetic abnormalities and their great effect on prognosis. About 30% of AML cases have a recurrent cytological abnormality which gives rise to a particular prognostic feature (Falini et al., 2011). Among the others some harbor mutations in various genes such as FLT3, NPM1 and CEBPA, each with a specific prognostic impact. In 2008 WHO classification of acute myeloid leukemias, NPM1 mutation, which leads to its cytoplasmic localization, has been introduced as a provisional subgroup with favorable prognosis (Hutchison and Schexneider, 2011). Its exact categorization as a new AML subgroup has been postponed to further scientific evidences.

Nucleophosmin (NPM) is a non-ribosomal nucleolar phosphoprotein with molecular weight of 35 to 40 KD (Lim and Wang, 2006). Through alternative mRNA splicing at least two isoforms of NPM; NPM1 and NPM1.2, are formed (Lim and Wang, 2006; Chang and Olson, 1990). During the cell cycle, it shuttles between the nucleus and cytoplasm (Lim and Wang, 2006; Borer et al., 1989; Szebeni, Herrera and Olson, 1995). NPM plays role in several cellular functions including ribosome biogenesis and centrosome duplication (Lim and Wang, 2006; Okuda et al., 2000). NPM over expression is reported in various malignancies such as tumors of colon, liver, stomach, ovary and prostate (Lim and Wang, 2006; Grisendi et al., 2006). Translocations involving NPM gene is commonly seen in hematopoetic malignancies (Morris t al., 1995; Redner et al., 1996; Yoneda et al., 1996). Furthermore aberrant cytoplasmic expression of NPM, followed by mutation, has been found in about one third of adult AMLs (Lim and Wang, 2006; Falini et al., 2005). It is still unclear that whether this cytoplasmic expression of NPM is associated with carcinogenesis (Lim and Wang, 2006). The role of NPM in carcinogenesis is controversial and both tumor suppressive and oncogenic functions have been reported. Unrestricted centrosome duplication as a result of NPM1 inactivation which leads to genomic instability, implies a possible tumor suppressive role for NPM (Lim and Wang, 2006). Effect on TP53 stabilization by blocking HDM2-TP53 complex, has also been reported as another explanation (Kurki et al., 2004). By contrast, its oncogenic role may be associated with activating the oncogenic potential of the fused proteins (e.g. ALK, RARA and MLF1) during translocations (Lim and Wang, 2006; Morris t al., 1995; Redner et al., 1996; Yoneda et al., 1996). In addition, NPM over expression leads to resistance to apoptosis and also it has been shown that NPM blocks ARF-mediated TP53 activation (Li et al., 2004; Wu, Chang and Yung, 2002; Korgaonkar et al., 2005).

P27 (CDKN1B) is a CDK inhibitor (CDKI) that prevents cell cycle progression through interaction with various CDKs. CDKIs are frequently disabled in human malignancies. Under expression of P27 has been associated with poor prognosis in various malignancies including breast cancer, colorectal cancer, gastric cancer, prostate cancer and lymphoma. In addition, P27 expression has been related to disease free survival in AML (Stricker and Kumar, 2010; Yokozawa et al., 2000).

NPM1 and P27 expression and their association with various clinicopathological features of the disease including survival, in AML cases, are studied in this research, attempting to elucidate their impact on prognosis and behavior of the disease.

Materials and Methods

Patients and samples

A total number of 30, histologically confirmed, AML cases that had underwent bone marrow aspiration (BMA) and bone marrow biopsy (BMB) between 2010 to 2013 in Quaem university hospital, enrolled the study. Their BMA and BMB slides were reviewed by two pathologists. Cases with inadequate specimen or non-diagnostic myeloperoxidase staining were excluded. Contacting patients or their families trough phone, survival of patients were surveyed. The investigation confirms the principles outlined in Helsinki Declaration and the study protocol was approved by the Research Ethics Committee in Mashhad University of Medical sciences. Patients' anonymity was preserved during the study.

IHC Staining

Paraffin embedded tissue samples were stained immunohistochemically for P27 and NPM1 markers, using Abcam kits (Anti-mutant Nucleophosmin antibody-carboxy terminal end ab 65816 and Anti-P27 KIP1antibody ab 32034), according to the manufacture's instruction, briefly as follows:

- Deparaffinization and hydration
- Washing with deionized water
- Antigen retrieval by 12 min incubation with 1% Molar Citrate buffer (PH=6) in a microwave oven
- Cold tap water washing for 10 min
- Ten min with 3% H2O2
- Overnight incubation with antibodies against NPM1 or P27 (primary antibodies) at 4^{oC}
- Rinsing with Triss buffer (2 x 5 min)

- Sixty min incubation with biotin linked anti-mouse and anti-rabbit immunoglobulins (secondary antibodies) at room temperature.
- Rinsing with Triss buffer (3 x 5 min)
- Ten min with Streptavidin-HRP (ab 7403) at room temperature
- Ten min with diamino benzidine (DAB) (ab 5814) at room temperature
- Rinsing in running tap water for 5 min
- Counter staining with Mayer's haematoxyline
- Rehydrating and mounting

Colorectal and breast cancer tissues were used as positive controls for NPM1 and P27, respectively. Excluding the primary Ab step during IHC staining, the negative controls were formed.

Results interpretation

The samples were considered to be positive for cytoplasmic NPM1 (cNPM1) if there was at least 10% cytoplasmic immunoreactivity in myeloblasts (according to Lua et al study (Luo et al., 2010)). Thirty percent nuclear (or cytoplasmic) immunoreactivity, was interpreted as P27 Positivity (according to Erlanson et al study (Erlanson et al., 1998)).

Statistical Analysis

Data analysis was performed using SPSS version 16. After checking for normal distribution of quantitative variables (using one-sample Kolmogrov-Smirrnov test), one-sample t-test, Chi-square test, log-rank test, and Kaplan-Meier survival curve were employed. Differences were considered statistically significant at $P<0.05$.

Results

Eighteen male and 12 female AML cases with a mean age of 36.5 years (ranging between 15 and 78 years), were participated the study. 29% of cases showed IHC immunoreactivity for cNPM1 and 23% were positive for P27. There was a significant relation between cytoplasmic NPM1 as well as P27 positivity and WBC count of more than 10,000/µl ($P=0.01$ and $P=0.033$ for cNPM1 and P27, respectively). There were not any statistically significant relation between cytoplasmic NPM1 and P27 expression and age, Hemoglobin, RBC count and Platelet count (tables 1 and 2).

The mean survival of our patients was 9 months (95% confidence interval: 7.5 – 10.6 months). The mean survival of cNPM1 positive cases was 9.1 months (95% confidence interval: 6.2-11.9 months) and for P27 positive cases was 9.8 months (95% confidence interval: 7.1-12.5 months). There was no significant difference between survival of cNPM1 positive and negative cases ($P>0.99$). There was no significant difference in survival of P27 positive and negative cases ($P>0.99$). Using Kaplan-Meier survival function and log-rank test, there were no significant relation between cNPM1 positivity and survival ($P=0.97$). Although the under curve area in P27 positive cases was more than P27 negative cases by Kaplan-Meier method, log-rank test showed no significant difference between survival of two groups ($P=0.69$) (Figure. 1).

In cNPM1 positive group of patients, the survival was longer in patients with platelet count of less than 50,000/µl, using Kaplan-Meier curve, but the difference was not statistically significant ($P=0.096$). The survival was also longer in patients with low platelet count (less than 50,000/µl) in P27 positive group, using Kaplan-Meier method ($P=0.058$).

Discussion

NPM is a nucleolar phosphoprotein that plays role in ribosome biogenesis and centrosome duplication (Lim and Wang, 2006; Okuda et al., 2000). NPM over expression is reported in various malignancies (Lim and Wang, 2006; Grisendi et al., 2006). Translocations involving NPM gene is commonly seen in hematopoetic malignancies (Morris t al., 1995; Redner et al., 1996; Yoneda et al., 1996). Furthermore aberrant cytoplasmic expression of NPM, followed by mutation, has been found in about one third of adult AMLs (Falini et al., 2005). It is still unclear that whether this cytoplasmic expression of NPM is associated with carcinogenesis (Lim and Wang, 2006). The role of NPM in carcinogenesis is controversial and both tumor suppressive and oncogenic functions have been reported. Unrestricted centrosome duplication as a result of NPM1 inactivation which leads to genomic instability, implies a possible tumor suppressive role for NPM (Lim and Wang, 2006). Its oncogenic role may be associated with activating the oncogenic potential of the fused proteins (e.g. ALK, RARA and MLF1) during translocations (Lim and Wang, 2006; Morris t al., 1995; Redner et al., 1996; Yoneda et al., 1996). In addition, NPM over expression leads to resistance to apoptosis and also it has been shown that NPM blocks ARF-mediated TP53 activation (Li et al., 2004; Wu, Chang and Yung, 2002; Korgaonkar et al., 2005). P27 is a CDK inhibitor (CDKI) that prevents cell cycle progression through interaction with various CDKs. CDKIs are frequently disabled in human malignancies. Under expression of P27 has

been associated with poor prognosis in various malignancies. In addition, P27 expression has been related to disease free survival in AML (Stricker and Kumar, 2010; Yokozawa et al., 2000).

In this research the BMB samples of 30 AML cases were stained immunohistochemically for cNPM1 and P27 markers. Twenty nine percent of cases showed cNPM1 positivity while 23% were immunoreactive for P27. The reported rate of cNPM1 positivity differs in various studies and ranging between 21% and 48% (Chauhan et al., 2013; Dohner et al., 2005; Dhahir and Dhahi, 2010). It may be related to the use of dissimilar methods (e.g. PCR and IHC) with different sensitivities (Lit BM, Kwong YL and Wong, 2016) as well as employing different cut off points. Some studies have found higher rates of cNPM1 expression in AML patients older than 35 years (Chauhan et al., 2013; Verhaak et al., 2005). We found higher rates of cNPM1 expression in patients younger than 35 years (55.6% of our positive cases were less than 35 years old). In line to our report Konoplev et al found more frequency of cNPM1 expression in younger than 35 years old patients (Coons, Creech and Jones, 1941). Further studies with larger sample size may elucidate the age related distribution of cNPM1 expression. P27 was positive in 23% of our cases, while Yokozawa et al, using PCR, found P27 expression in 48.6% of their patients (Yokozawa et al., 2000). This difference could be related to higher sensitivity of PCR comparing IHC.

cNPM1expression in this study was associated with higher (>10,000/µl) WBC count (P=0.01). In line to our finding Dohner et al and Verhaak et al also reported a relation between cNPM1 expression and higher WBC count (Dohner et al., 2005; Verhaak et al., 2005). As NPM over expressed cells are resistant to apoptosis following some kinds of cellular stresses, this finding can be explained (Li et al., 2004; Wu, Chang and Yung ,2002). We also found a relation between P27 expression and WBC count of more than 10,000/µl (P=0.033). Similar to our research, Markaki et al showed a significant relation between P27 expression and higher WBC count (p=0.01) (Markaki et al., 2006). In contrast Yokozawa et al did not find a relation between P27 and WBC count (Yokozawa et al., 2000). The differences between studies could be associated to limited sample sizes. Therapy related decrease in WBC count in the last study may be another explanation.

We did not find any significant relation between cNPM1 or P27 expression and blood hemoglobin or hematocrit, which is in line with other studies (Dhahir and Dhahi, 2010; Markaki et al., 2006).

In this study there was no difference in survival of patients with or without cNPM1 expression. In the other studies the favorable prognostic effect of cNPM1 expression has been found in AML cases with normal karyotype and without FLT3 mutation (Falini et al., 2011; Hutchison and Schexneider, 2011). Finding no favorable effect on survival in our study may be related to the fact that karyotypic status and the presences of FLT3 mutation in our samples have not been examined. Further studies with larger sample sizes and studies that investigate the presence of various mutations and karyotypic abnormalities simultaneously, are needed to elucidate the precise effect of cNPM1 expression on prognosis of AML.

In this study the survival of P27 positive patients were higher than P27 negative cases (10 months vs. 8.7 months), but it was not statistically significant (p=0.68). In line to our study Zolotta et al and Markaki et al did not find any relation between P27 expression and survival of AML patients (Markaki et al., 2006; Zolota et al., 2007). In contrast, Yokozawa et al reported a significant relation between low P27 expression and poor prognosis in AML, which is in concordance with prognostic impact of P27 in other malignancies (Yokozawa et al., 2000). Finding no significant relation between P27 and survival in our research may be associated to small size of sampling.

cNPM1 and P27 expression rates and their prognostic impact have been studied in various types of malignancies, although their precise effect on prognosis of AML have not been clarified yet. In this study cNPM1 and P27 expressions were not associated with survival in AML patients. As favorable prognostic effect of NPM1 mutation was seen in AML cases that have normal karyotype and don't harbor FLT3 mutation (Falini et al., 2011; Hutchison and Schexneider, 2011), our findings may emphasizes that prognostic effect of NPM1 mutation is related to other genetic abnormalities present in the cell. Further studies with larger sample sizes and simultaneous examination of various genetic and cytogenetic abnormalities may be helpful in clarifying the exact role of these markers in pathogenesis of AML and their prognostic impact on the disease.

Conflict of Interest:

The authors declare that they have **no** conflict of interests.

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Table 1. Comparisons of cNPM1 and P27 expression between different subgroups of patients.

	cNPM1 Expression		P Value	P27 Expression		P Value
	Present	Absent		Present	Absent	
Gender						
Male	66.7% (6)	57.1% (12)	0.7	71.4% (5)	56.5% (13)	0.66
Female	33.3% (3)	42.9% (9)		28.6% (2)	43.5% (10)	
Lymph Node Involement						
Present	33.3% (3)	23.8% (5)	0.66	42.9% (3)	21.7% (5)	0.34
Absent	66.7% (6)	76.2% (16)		57.1% (4)	78.3% (18)	
Hepatomegaly						
Present	11.1% (1)	23.8% (5)	0.63	0% (0)	26.1% (6)	0.29
Absent	88.9% (8)	76.2% (16)		100% (7)	73.9% (17)	
WBC Couts						
≥ 10000 /µl	100% (9)	52.4% (11)	**0.01**	100% (7)	56.5% (13)	**0.033**
< 10000 /µl	0% (0)	47.6% (10)		0% (0)	43.5% (10)	
Blood Hemoglobin						
≥ 10 /dl	88.9% (8)	100% (21)	0.31	100% (7)	95.7% (22)	0.88
< 10 /dl	11.1% (1)	0% (0)		0% (0)	4.3% (1)	
Platelet Counts						
≥ 50000 /µl	55.6% (5)	52.4% (11)	0.96	28.6% (2)	6.19% (14)	0.16
< 50000 /µl	44.4% (4)	47.6% (10)		71.4% (5)	39.1% (9)	

Numbers in parentheses represents number of cases. cNPM1: Cytoplasmic Nucleophosmin

Table 2. Comparisons of cNPM1 and P27 expression and clinical and laboratory findings of patients.

	cNPM1 Expression		P Value	P27 Expression		P Value
	Present	Absent		Present	Absent	
	Mean ± SD†	**Mean ± SD†**		**Mean ± SD†**	**Mean ± SD†**	
Age (years)	38.4 ± 15.5	35.7 ± 19.9	0.71	32.6 ± 14.6	37.6 ± 19.7	0.55
WBC Counts(/µl)	46577 ± 2689	31080 ± 4734	**0.01**	54171 ± 5411	30117 ± 3781	**0.033**
RBC Counts (/µl)	3494400 ± 408078	3219000 ± 424993	0.11	3521400 ± 195485	3234800 ± 464767	0.12
Blood Hemoglobin (/dl)	8.14 ± 2	7.45 ± 1.5	0.31	7.74 ± 1.2	7.63 ± 1.8	0.88
Platelet Count (/µl)	48888 ± 5578	49809 ± 4178	0.96	70428 ± 5240	43173 ± 4229	0.16

†Mean ± SD: Mean ± Standard Deviation
cNPM1: Cytoplasmic Nucleophosmin

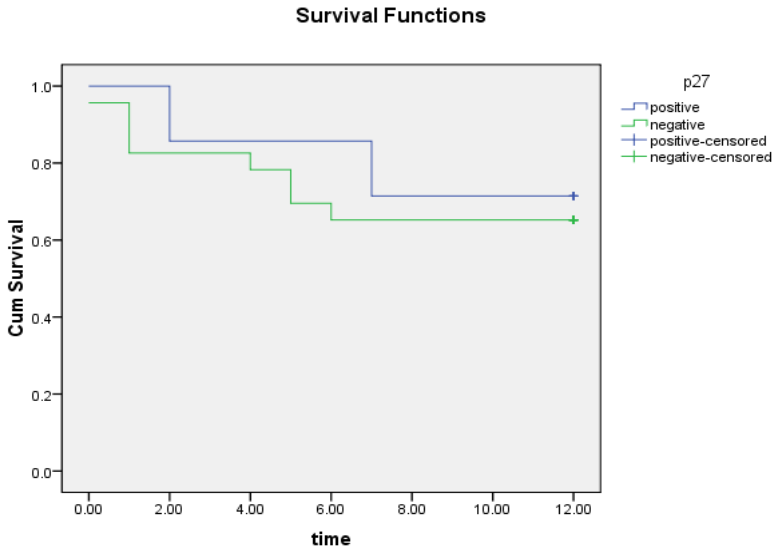

Figure 1. Kaplan-Meier survival functins for P27 positive and negative AML patients. Although the under curve area in P27 positive cases was more than P27 negative cases, the differences were not statistically significant.