Original Article

The level of bone marrow WT1 message is a useful marker to differentiate myelodysplastic syndromes with low blast percentage from cytopenia due to other reasons

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COI

The authors state that they have no Conflict of Interest (COI).

Abstract

Objective Myelodysplastic syndromes (MDS) are a group of hematological neoplasms associated with ineffective hematopoiesis and that transform to acute leukemia. Distinguishing MDS from other cytopenias is sometimes difficult even for trained hematologists. WT1, the gene mutated in Wilms' tumor, was found expressed in acute myeloid leukemia (AML) and MDS. The amount of WT1 in peripheral blood and bone marrow (BM) is low in low-risk MDS subtypes, and is high in high-risk MDS subtypes. However, the role of WT1 in the differential diagnosis between MDS and other diseases showing cytopenia has not been fully addressed. The present study evaluated whether WT1 expression level can assist in the differential diagnosis of MDS from other cytopenias.

Methods The amount of WT1 message was evaluated among 56 MDS patients and 47 patients with cytopenia for various other reasons (cytopenia VR) at the Nagasaki University Hospital.

Results The level of WT1 was significantly related to the percentage of blasts in BM among MDS cases, and the type of FAB classification of MDS; refractory anemia (RA) cases showed significantly lower WT1 level than patients with RA with excess blasts. WT1 level was significantly related to the prognostic risk categories of MDS by the International Prognostic Scoring System (IPSS) and the revised IPSS. Although the blast percentage in the BM of RA and cytopenia VR were both less than 5%, there was a significant difference in the level of WT1 between MDS and cytopenia VR.

Conclusion WT1 might be a good marker to differentiate low blast percentage MDS and cytopenia VR.

Key words: myelodysplastic syndromes, WT1, cytopenia, IPSS, IPSS-R

Introduction

Myelodysplastic syndromes (MDS) are a group of hematological neoplasms associated with ineffective hematopoiesis and that can transform to acute leukemia [1, 2]. MDS is primarily a disease of the elderly, and the number of patients with MDS is expected to increase along with the aging of the society in Japan. Although there are many recent reports regarding genetic alterations in MDS [3-7], the detailed etiology of this disease has not been fully elucidated. For the diagnosis of MDS, it is necessary to count immature blasts in peripheral blood (PB) or bone marrow (BM) smears and to evaluate dysplasia of hematopoietic cells [8]. French-American-British (FAB) classification and World Health Organization (WHO) classification are widely used for the diagnosis and classification of MDS cases [1, 2]. Distinguishing MDS from other cytopenias, such as secondary anemia and aplastic anemia can be performed by using clinical and hematological data and by assessing the morphology of hematopoietic cells. However, in clinical practice, it is sometimes difficult even for trained hematologists to diagnose MDS.

Lower risk MDS, which comprises 70% of MDS cases, shows low blast percentage in BM, and its dysplastic features of hematopoietic cells tend to be mild. As a result, some patients must be followed for months before a final diagnosis is achieved. Therefore, a good diagnostic marker other than morphology would be useful for the diagnosis of MDS in patients with low blast percentage or mild dysplasia. Such difficulty in diagnosis occurs, for example, in cases of hypoplastic MDS. MDS with hypoplastic bone marrow cellularity comprises about 10% of all MDS cases [2], and such cases might be more frequent among Japanese MDS cases. Because both low cellularity and morphologically mild dysplasia are present in these cases, it is difficult to differentiate hypoplastic MDS from mild aplastic anemia.

WT1, the gene mutated in Wilms' tumor, was found expressed in acute myeloid leukemia (AML), and the level of its expression was reported to be associated with minimal residual disease of AML after chemotherapy [9, 10].

Since WT1 level is very high at the time of AML diagnosis, PB is widely used for this purpose, though BM samples usually show about 10 times higher WT1 level than PB samples. Recent reports suggest that WT1 is expressed in and reflects the disease status of MDS [11]. The amount of WT1 in PB and BM is low in lower risk MDS subtypes, such as refractory anemia (RA) by the FAB classification, and is high in those with higher risk MDS subtypes, such as refractory anemia with excess blasts (RAEB). This suggests that WT1 level could be a prognostic marker for patients with MDS. However, the role of WT1 in the differential diagnosis between MDS and other diseases showing cytopenia has not been fully addressed.

The present study evaluated whether WT1 expression level can assist in the differential diagnosis of MDS from other hematological states with cytopenias. For this purpose, we measured the level of WT1 message using quantitative polymerase chain reaction (PCR) assay for BM samples.

Patients and Methods

Patients and diagnosis

From February 2009 to August 2013, 56 patients with a diagnosis of MDS and 47 patients with certain levels of cytopenia for various other reasons (cytopenia VR) were enrolled in this study after obtaining informed consent (IRB approval number 14062381). Cytopenia was defined as follows: anemia, hemoglobin (Hb) < 10 g/dL; neutropenia, neutrophil count < 1800/μL; and thrombocytopenia, platelet count (PLT) < 1 x 10^5/μL.

All patients were diagnosed at the Department of Hematology of Nagasaki University Hospital. For the diagnosis of MDS and other diseases, at least two hematologists evaluated hematological data, BM and PB smears independently. Final diagnosis of each case was determined by three hematologists (M. B., T. H., and Y. M.) following FAB classification and WHO classification. Of note, MDS with low blast percentage (< 5% in BM) is classified into RA in the FAB classification and as refractory cytopenia with uni- or multi-lineage dysplasia (RCUD or RCMD) in the WHO classification. MDS cases were risk stratified using the international prognostic scoring

system (IPSS) [12] and the revised IPSS (IPSS-R) [13]. IPSS divides MDS cases into four risk groups (low, intermediate-1, intermediate-2, and high) using three factors (number of cytopenic lineage, cytogenetic category for IPSS, and blast percentage in BM). IPSS-R is a revised version of IPSS that has five risk categories (very low, low, intermediate, high and very high) using similar factors as those used for IPSS (level of cytopenia in each lineage, cytogenetic category for IPSS-R, and blast percentage in BM). Among cases with cytopenia VR, there were three cases with idiopathic cytopenia of undermined significance (ICUS) [14] that showed minimal levels (less than 10%) of dysplastic changes in hematopoietic cells of PB and BM. There were also 12 cases of secondary cytopenia with various underlying diseases and five cases of reactive cytopenia that recovered after a certain period of time.

Extraction of total RNA and cDNA synthesis from BM samples

After hemolysis using NH_4Cl (50 mM), cells were washed three times with phosphate-buffered saline. Then, total RNA was extracted using the PureLink RNA Micro Kit (Invitrogen, Carsland, CA, USA) following the manufacturer's recommendation. Oligo (dT) primer and Super Script III Reverse Transcriptase (Invitrogen) were used to generate cDNA.

Quantification of WT1 mRNA

After cDNA was prepared, the amount of WT1 message was evaluated using PCR via a LightCycler 480 (Roche Diagnostics, Indianapolis, USA) following the instructions [15]. The primers used for the quantification of WT1 message were: 5'-GATAACCACACAACGCCCATC-3', and 5'-CACACGTCGCACATCCTGAAT-3'. The sequence of TaqMan probe was 5'-ACACCGTGCGTGTGTATTCTGTATTGG-3'. PCR was performed in 45 cycles of 95°C for 10 seconds and 60°C for 30 seconds. WT1 cDNA cloned into TA vector (7.44×10^6 copies/2 μL) was used as standard for the reaction. The final data are shown as WT1 message copies/μg total RNA.

Statistical analysis

The Pearson's correlation coefficient test was done for analysis of correlation between WT1 mRNA and blast percent in bone marrow. Receiver operating characteristics (ROC) analysis was performed to determine diagnostic accuracy of the level of WT1. For comparison of the number of WT1 message between groups, a Mann-Whitney *U* test and a Wilcoxon rank-sum test was performed. The difference was thought significant when the P value was less than 0.05. Statistical analysis was performed using GraphPad Prism 6.01 (GraphPad Software, La Jolla, CA, USA).

Results

Characteristics of patients with MDS and with cytopenia VR

There were 56 patients with MDS including nine patients with hypoplastic MDS (Table 1). Thirty-five cases were diagnosed as having RA, one case was diagnosed with RA with ring sideroblasts (RARS), and 20 cases were diagnosed with RAEB by FAB classification. By WHO classification, six patients had RCUD, one patient had RARS, 23 patients had RCMD, 11 patients had RAEB-1, nine patients had RAEB-2, and six patients had MDS unclassifiable (MDS-U). Because the number of cases in each WHO classification is small, especially in RCUD, RAEB-2, and MDS-U, we used the FAB classification for further analysis. In terms of the IPSS risk category, seven patients were classified as "Low", 33 patients were classified as "Intermediate-1 (Int-1)", 10 patients were classified as Int-2, and six patients were classified as "High" risk. For IPSS-R, three patients were classified as "Very low", 12 patients were classified as "Low", 23 patients were classified as "Int", eight patients were classified as "High", and 10 patients were classified as "Very high" risk.

Forty-seven cases of cytopenia VR were also included in this study (Table 2). The median age of patients with cytopenia VR was 70 years, which was younger than that of the MDS cases (74 years). In the cytopenia VR group, three cases were idiopathic cytopenia of undetermined significance (ICUS)

[14], which show dysplastic features in hematopoietic cells but not to a degree sufficient to diagnose MDS. Aplastic anemia (AA, nine cases), idiopathic thrombocytopenic purpura (ITP, five cases), Evans syndrome (two cases), other immune-related causes (Sjogren's syndrome, etc., two cases), and pure red cell aplasia (PRCA, one case) were also in the cytopenia VR group. Among them, ITP, Evans syndrome, PRCA, and other immune-related cases were grouped as the immune-related causes (IR) group. Seventeen cases of "others" included those who had malignancies, liver cirrhosis, renal dysfunction, splenomegaly of unknown cause, or infections, and these cases were categorized as secondary and reactive (SR).

Levels of WT1 message in MDS cases

The copy number of WT1 message varied widely among cases of MDS. The median value was 6931 copies/μ g RNA with a range of 76–201,500 copies/μ g RNA. The level of WT1 was significantly related to the percentage of blasts in the BM smear among MDS cases (Table 3 and Figure 1; r= 0.3678, P = 0.0053), but was not related to the karyotype groups according to the IPSS-R category. The level of WT1 was also significantly different according to the type of FAB classification. RA cases whose blast percentage was less than 5% in BM showed significantly lower WT1 level when compared with patients with RAEB who had ≥ 5% of blasts in the BM (Table 3, median WT1 values were 6843 copies/μ g RNA and 30700 copies/μ g RNA, respectively; P= 0.0008). Interestingly, WT1 level was significantly related to the prognostic risk categories of MDS by IPSS and IPSS-R. As shown in Figure 2, among IPSS categories, Low + Int-1 (lower risk group) had significantly lower WT1 level than Int-2 + High (higher risk group) (P=0.0136). A significant relationship between WT1 level and the prognostic risk groups was also observed for IPSS-R categories. Although the WT-1 level of each IPSS-R category did not show clear differences (Table 3 and Figure 3A), the amount of WT1 was significantly related among three groups: Very low + Low, Int, and High + Very high groups (Table 3 and Figure 3B; P = 0.0326). Based on

this observation, we assumed that the number of cases in each IPSS-R category was not sufficient to demonstrate a significant difference in the level of WT1 (Figure 3A). These data suggested that the level of WT1 among MDS was related to the disease characteristics, such as blast percentage of BM, disease subtype by FAB classification, and also prognostic categories defined by the scoring systems for MDS (IPSS and IPSS-R).

WT1 message levels in cytopenia VR, and comparison with MDS

The median copy number of WT1 message was 927.2 (range, 10.7–6458) in cytopenia VR (Table 3). The median value of WT1 in AA, SR, IR, and ICUS were 958.7, 917.1, 852.6, and 927.2 copies/μ g RNA, respectively. We did not find significant differences in these values among subgroups of cytopenia VR (data not shown). Including ICUS, no case in cytopenia VR had BM blasts at more than 5%. There was a significant difference in the WT1 level between MDS and cytopenia VR (Table 3 and Figure 4A; median value: 6931 and 927.2, respectively; $P<0.0001$). Although the blasts in the BM of RA and cytopenia VR were both less than 5%, there was also a significant difference in the level of WT1 between these two categories (Table 3 and Figure 4B; median value: 3843 copies/μ g RNA and 927.2 copies/μ g RNA, respectively; $P < 0.0001$). Furthermore, there was a significant difference between hypoplastic MDS and aplastic anemia (Table 3, $P = 0.0048$), and between MDS and normo / hypercellular cytopenia VR (Table 3, $P < 0.001$).
In order to evaluate the diagnostic potential of the WT1 mRNA, ROC curve analysis was performed. The area under the curve (AUC) was 0.80091 (Figure 5). The sensitivity and the specificity to diagnose MDS was 76.8% and 78.7%, respectivery. The cut off value of WT1 mRNA was 2040 copies/μ g RNA.

Discussion

In this study, we measured the WT1 mRNA level in the BM of cytopenic

patients, including patients with MDS and cytopenia with various underlying diseases. We used BM as sources to measure WT1 in order to directly assess whether the WT1 level reflected the hematopoietic situation of each patient. Indeed, the main purpose of this study was to evaluate the utility of WT1 mRNA to differentiate low blast MDS from cytopenias related to non-MDS diseases.

We found that the level of WT1 was significantly different between MDS and cytopenia VR, demonstrating that WT1 could be useful for the differential diagnosis of MDS. Importantly, WT1 level of RA was also significantly higher than that of cytopenia VR, suggesting its possible utility in the separation of low-blast MDS cases from non-MDS. We also confirmed results from previous reports that showed that the WT1 level of RAEB (usually in higher risk MDS) was significantly higher than that of RA (mostly in lower risk MDS) [11], supporting its prognostic role in MDS. Although we could not show a statistically different WT1 value according to each IPSS-R category (Table 3 and Figure 3A), we showed that combined groups of IPSS-R were significantly related to the level of WT1 (Table 3 and Figure 3B). It is possible that the limited number of cases would be one of the reasons for these results. Since treatment varied widely among patients, even among those in the same MDS subtype in this study, we could not evaluate the direct relationship between WT1 level and prognosis of each case. However, considering that both IPSS and IPSS-R have strong predictive value for MDS cases [12, 13, 16, 17], it is possible that WT1 could be used for the prediction of prognosis.

On the other hand, WT1 levels of cytopenia VR did not show much variation in each category as was otherwise seen among MDS cases, which probably resulted in a lower value of WT1 in the differential diagnosis among cytopenia VR cases. Since MDS RA showed relatively high WT1 value, it seems that blast percentage is not the only factor that would determine WT1 level. Future studies should investigate how WT1 level is regulated in different situations, especially in hematopoietic cells of MDS.

In conclusion, we demonstrated that the level of WT1 was significantly associated with the FAB subtype of MDS, and that WT1 might be a good

marker to differentiate low blast percentage MDS and cytopenia caused by various reasons.

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Figure legends

Figure 1. Relationship between WT1 level and blast percentage in BM among MDS patients
WT1 levels in BM were positively related with the percentage of blasts in BM with statistical significance (P = 0.0053).

Figure 2. WT1 levels by combined IPSS category
WT1 level of the lower risk group (Low and Int-1) was significantly lower than that of higher risk group (Int-2 and High) (P = 0.0136).

Figure 3A. WT1 levels by IPSS-R category

Figure 3B. WT1 levels by combined IPSS-R category
WT1 level in each IPSS-R category (Very low, Low, Intermediate, High, and Very high) did not show statistically significant difference as a whole (Figure 3A). However, combined categories (Very low + Low, Intermediate, High + Very high) showed difference with significance (Figure 3B; P = 0.0326).

Figure 4A. Comparison of WT1 levels between MDS and cytopenia for various other reasons.

Figure 4B. WT1 levels by FAB classification and cytopenia for various other reasons.
WT1 level of cytopenia for various other reasons was significantly lower than that of MDS (Figure 4A; P < 0.0001), and that of each FAB subtypes of MDS (Figure 4B; RA and RAEB, both P < 0.0001).

Figure 5. Receiver operating characteristics (ROC) analysis of the WT1 level to distinguish between MDS and cytopenia VR. The area under the curve (AUC) was 0.80091. The sensitivity and the specificity to diagnose MDS was 76.8% and 78.7%, respectively.

Table 1. Clinical characteristics of myelodysplastic syndromes patients (n = 56).

Characteristics	No. of patients
Median age(range)	74 (42-90)
Male / Female	41 / 15
FAB subtypes	
RA	35
RARS	1
RAEB	20
WHO subtypes	
RCUD	6
RARS	1
RCMD	23
RAEB-1	11
RAEB-2	9
MDS-U	6
cellularity	
hypoplastic	6
normocellular	27
hypercellular	23
karyotype	
normal	26
del(20)(q11.2)	7
del(7q) / -7	5
trisomy 8	4
complex	3
other	11
IPSS	
Low	7
Intermediate-1	33
Intermediate-2	10

High	6
IPSS-R	
Very low	3
Low	12
Intermediate	23
High	8
Very high	10

FAB, French-American-British; RA, refractory anemia; RARS; RA with ring sideloblasts; RAEB, RA with excess blasts; RCUD, refractory cytopenia with uniliniage dysplasia; RCMD, refractory cytopenia with multilineage dysplasia; MDS-U, MDS unclassifiable; IPSS, International Prognostic Scoring System; IPSS-R, revised IPSS.

Table 2. Clinical characteristics of patients with cytopenia for various other reasons (n = 47).

Characteristics	No. of patients
Median age, range	70, 23-89
Male / Female	24 / 23
Diagnosis	
AA	9
PRCA	1
ITP	5
Evans syndrome	2
Immunological	2
IDA	2
megaloblastic anemia	2
ICUS	3
Drug induced	4
others	17

AA, aplastic anemia; PRCA, pure red cell aplasia; ITP, idiopathic thrombocytopenic purpura; IDA, iron deficiency anemia; ICUS, idiopathic cytopenia of undetermined significance.

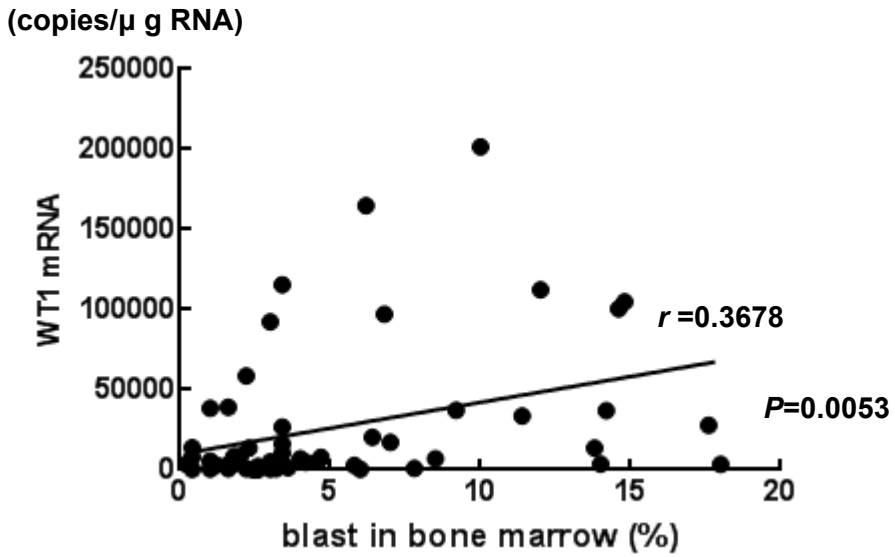
Table 3. Copy number of WT1 in various groups of MDS and cytopenia VR.

	Category (n)	Median (copies/μ g RNA)	Range (copies/μ g RNA)	P value
Diagnosis group	Cytopenia VR(47)	927.2	10.7 - 6458	
	MDS(56)	6931	76 - 201500	P < 0.0001
	Cytopenia VR(47)	927.2	10.7 - 6458	
	RA (35)	3843	76 - 92400	P = 0.0003
	AA (9)	958.7	57.2 - 3783	
	Hypoplastic MDS (6)	16912	1313 - 112400	P = 0.0048
	MDS(56)	6931	76 - 201500	
	Normo/ hypercellular Cytopenia VR(23)	927.2	299.3 -6458	P < 0.0001
FAB subtypes	RA (35)	3843	76 - 92400	
	RAEB (20)	30700	268 - 201500	P = 0.0008
WHO subtypes	RCUD (6)	2118	610 - 92400	
	RCMD(23)	5093	76 - 58700	
	RAEB-1(11)	20270	268 - 201500	
	RAEB-2(9)	33570	3528 - 112400	
	MDS-U(6)	3392	292 - 13760	P = 0.0244
cytogenetics (IPSS-R)	Good(34)	6931	76-201500	
	Intermediate(12)	6027	252-164900	
	Poor(6)	5324	140-104900	
	Very poor(4)	50365	2688-100500	N.S.
IPSS-R	Very low + Low(15)	3475	76 - 58700	

	Intermediate(23)	6878	292- 115500	
	High + Very high(18)	22475	140 - 201500	P = 0.0326

N.S., not significant.

Figure 1.


(copies/μ g RNA)
250000
200000
150000
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50000
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WT1 mRNA
r =0.3678
P=0.0053
0
5
10
15
20
blast in bone marrow (%)

Figure 2.

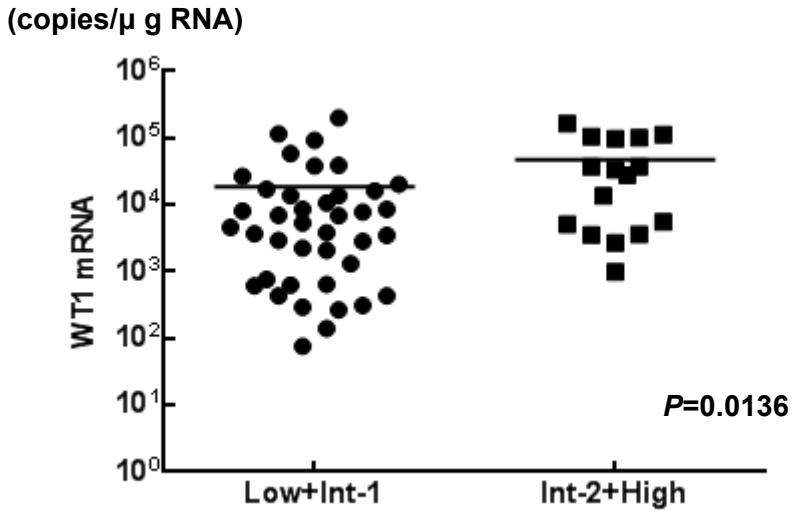
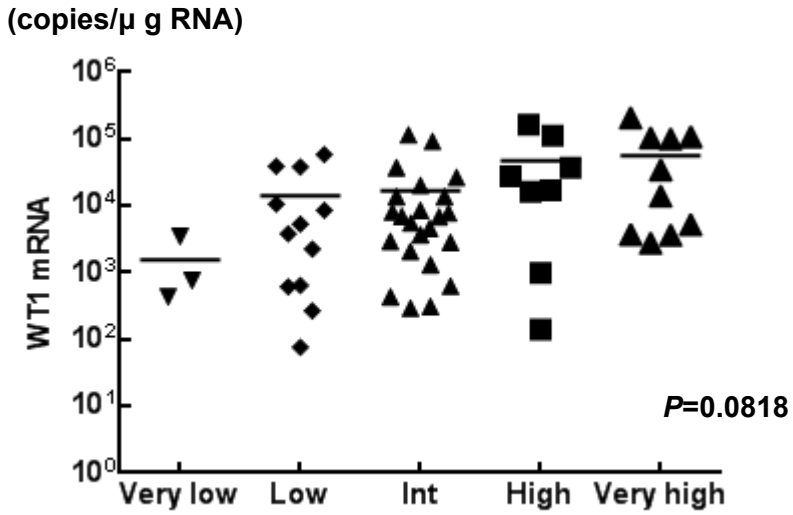

(copies/μ g RNA)
WT1 mRNA
10^6
10^5
10^4
10^3
10^2
10^1
10^0
P=0.0136
Low+Int-1
Int-2+High

Figure 3A



Figyre 3B

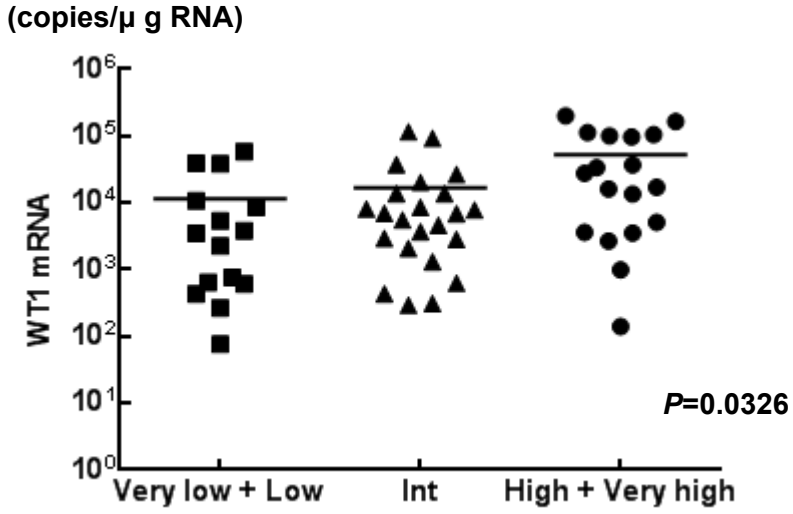


Figure 4A

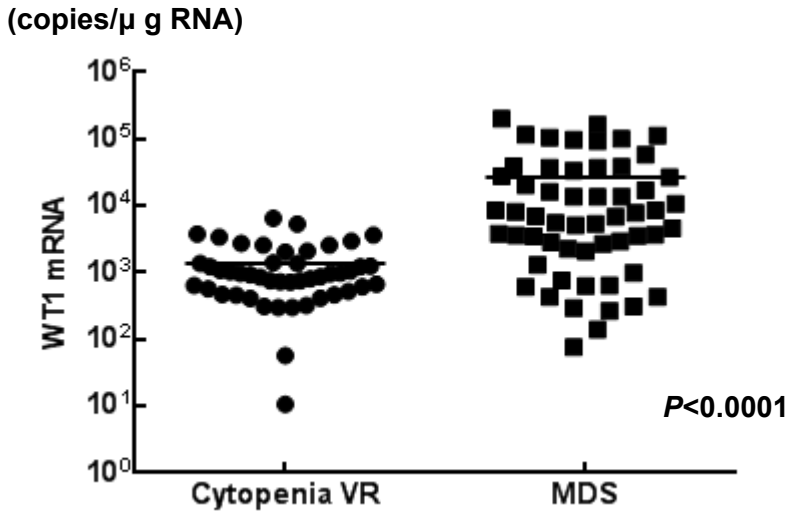


Figure 4B

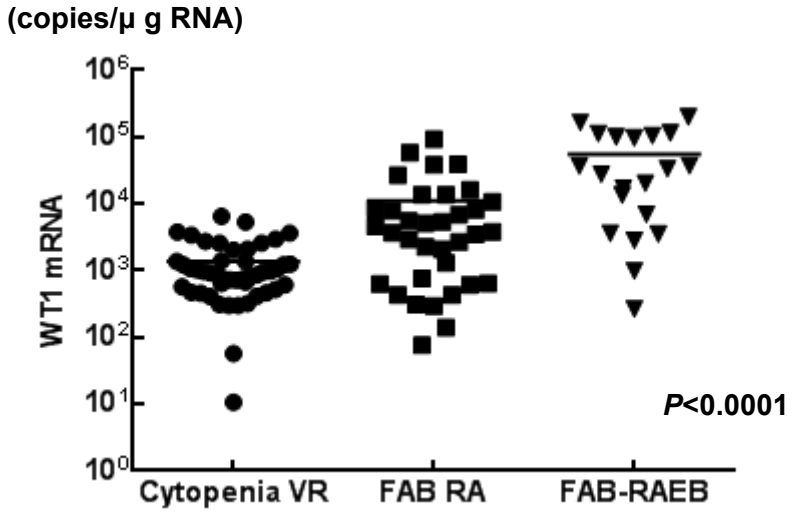
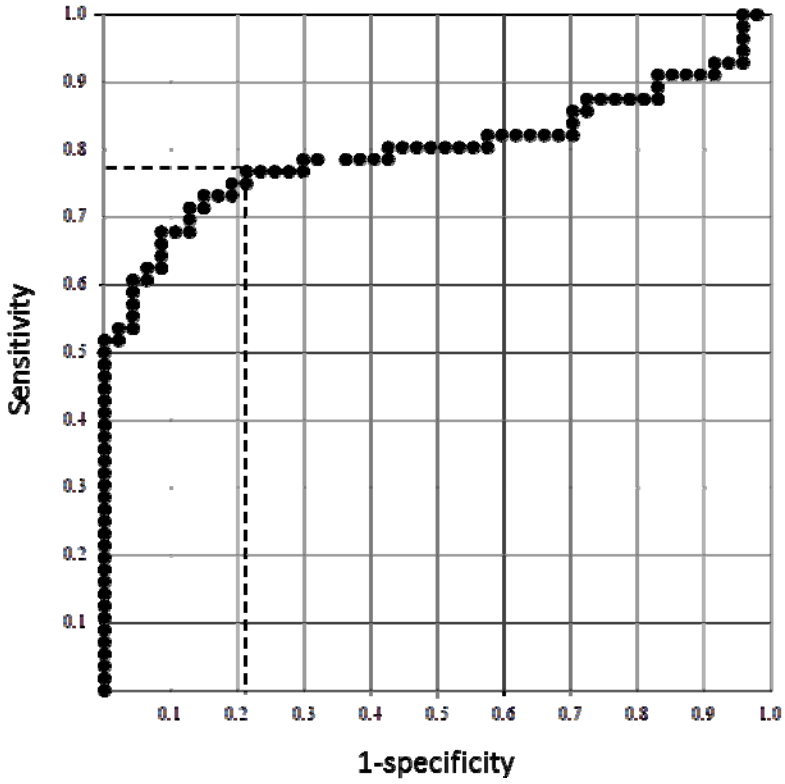


Figure 5


Sensitivity
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