



Letter to the Editor

Surface plasmon resonance analysis to detect the β^+ IVSI-110 thalassemia mutation in circulating cell-free fetal DNA



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To the Editor:

Biosensor technology based on surface plasmon resonance (SPR) and automated systems such as Biacore™ instruments, is an interesting tool for the real-time study of biomolecular interactions, including the identification of point mutations related to human genetic diseases as cystic fibrosis and β thalassemia [1,2].

Moreover, we have recently reported the detection of Y-chromosome sequences in circulating cell-free fetal (cff) DNA analyzing plasma samples obtained from 26 pregnant women at different weeks of gestation using Biacore™ X100, identifying with high accuracy Sex-determining Region Y (SRY) sequence of male fetuses in samples at early gestational age (6th–7th week), not usually detectable by quantitative Real-Time PCR, suggesting the possibility of non-invasive early fetal gender determination using cffDNA in combination with SPR-based biosensors [3].

Since the discovery of cffDNA in maternal plasma [4], non-invasive prenatal diagnosis has been assayed including the investigation of monogenic disorders [5], trying to manage the very low amount of fetal DNA sequences obtainable from maternal plasma [6].

So we have focused on the possible application of the developed SPR-based techniques as a non-invasive diagnostic tool to identify genetic point mutations such as the β^+ IVSI-110 responsible of a β^+ thalassemia phenotype, characterized by reduction of the β globin chains of adult hemoglobin and severe anemia.

The β^+ IVSI-110 mutation consists of the replacement of a guanine with an adenine in the first intron of the β globin gene, creating an alternative intronic splicing site causing the abnormal processing of 80–90% of pre-mRNAs [7].

This non-invasive diagnostic strategy for the detection of the β^+ IVSI-110 thalassemia mutation inherited from the father, was applied to blood samples obtained from pregnant women at different gestational ages after informed consent and with the approval of Ethical Committee of S. Anna Hospital Ferrara, Italy. Plasma samples were prepared according to the protocol described in the literature [8]. The total cell-free circulating DNA was extracted from 2 ml of maternal plasma by using the QIAamp® DSP Virus Spin Kit (Qiagen), as previously reported [3] and all experiments were conducted in agreement with the Declaration of Helsinki.

In order to confirm the father mutation or analyze the fetus genotype for the β^+ IVSI-110 thalassemia mutation, salivary swabs were collected, and genomic DNAs were extracted and sequenced as previously reported [9].

Three plasma samples from pregnant women were available with father carriers of β^+ IVSI-110 thalassemia mutation: #138, #140 and #207, collected at the 37th, 15th and 37th gestational week, respectively.

Moreover fetal genotypes were determined by sequencing the purified genomic DNA: only in one case (#140) the fetus had inherited the mutation from the father, resulting in a heterozygous N/ β^+ IVSI-110 genotype, but not in the other two cases (#138 and #207).

In order to obtain a detectable amount of PCR product for the hybridization with the immobilized probes on sensor chips, 5 μ l of the DNA template were subjected to a double pre-amplification reaction before the unbalanced PCR to produce single-stranded target sequences useful for the interaction with the probes. In fact a double pre-amplification of templates was required, as already demonstrated in the application of Biacore™ system for the non-invasive prenatal detection of the fetal sex [3]. In particular, the first balanced amplification of a 74 bp sequence was performed in a final volume of 50 μ l containing ExTaq Buffer (Takara) with 2 mM MgCl₂, 0.2 mM dNTPs, 150 ng PCR primers (IVS110BF, 5'-AGAGAAGACTCTGGGTTTCTGATAG-3' and IVS110BR, 5'-GCAGCCTAAGGGTGGGAAA-3'), and 1.25 U/reaction ExTaq DNA polymerase (Takara). The following amplification program was employed: 94 °C for 2 min; 50 amplification cycles comprising 94 °C for 30 s, 55 °C for 30 s and 72 °C for 10 s; 72 °C for 10 min.

For the double pre-amplification, a second identical balanced amplification was performed using 5 μ l of the first PCR product as a template. Finally, an unbalanced amplification was performed using 5 μ l of the balanced PCR product with only the forward primer IVS110BF at the same PCR conditions previously described.

The Biacore™ X100 analytical system (Biacore, GE Healthcare, Chalfont St Giles, UK) was used with SA sensor chips (Biacore, GE Healthcare), precoated with streptavidin, and the running buffer HBS-EP+ (Biacore, GE Healthcare). The experiments were conducted at 25 °C temperature and at 5 μ l/min flow rate. In order to immobilize the biotinylated DNA probes (normal β^N probe: 5'-biot-AGACCAATAGGC-3'; mutated β^M probe: 5'-biot-AGACTAATAGGC-3') on the SA sensor chip surface, the well-documented streptavidin-biotin interaction was employed [10]. After pretreatment with three 10 μ l pulses with 50 mM NaOH-1 M NaCl, an injection of 40 μ l of HBS-EP+ containing the oligonucleotide probe at the concentration of 10 ng/ μ l was administered on the sensor chip.

The analysis of biospecific interaction with unbalanced PCR products was carried out by injecting the target in HBS-EP+ buffer for 12 min, and then carrying out a 3 min washing step with the running buffer alone. After hybridization, the sensor chip was regenerated by performing a 5 μ l pulse of 50 mM NaOH. Sensorgrams were analyzed with the Biacore™ X100 Evaluation Software, version 2.0.1 (Biacore, GE Healthcare). Resonance unit (RU) values were measured after the analyte injection (RU_{in}) and the washing step (RU_{res}).

Table 1

List of mean final (RU_{fin}) and residual (RU_{res}) resonance unit values measured after three SPR-based analyses for each specimen of circulating DNA. The gestational age of the pregnant woman and the actual fetus genotype are also indicated for all the samples.

Sample	Gestational week	Fetus genotype	Probe	RU _{fin}	RU _{res}
#138	37	N/N	β^N	220	168
			β^M	12	4
#140	15	N/ β^+ IVSI-110	β^N	272	216
			β^M	39	15
#207	37	N/N	β^N	283	213
			β^M	18	6

As reported in Table 1, the mutated probe was able to produce, with the target PCR product obtained from sample #140 (carrying a heterozygous N/ β^+ IVSI-110 fetus), an hybridization signal higher than those of #138 and #207 samples, showing high binding specificity, despite the reduced amount of circulating fetal DNA compared to maternal DNA. In this case, a lower cell-free fetal DNA concentration was expected for sample #140 in comparison with samples #138 and #207, because the pregnant blood collection had been performed at an earlier gestational period.

In conclusion our data demonstrated the potential applicability of the developed SPR-based diagnostic approach to non-invasive prenatal diagnosis. Obviously, the validation as diagnostic tool in this field should require the analysis of a greater number of samples, possibly collected at various gestational ages to determine whether the fetal alteration can be identified at early gestational weeks. In addition, the non-invasive prenatal analysis of other point mutations causing β thalassemia or different genetic diseases should be performed.

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