Molecular diagnosis of Fanconi anemia with next-generation sequencing in a case with subtle signs and a negative chromosomal breakage test

Deniz Aslan[1], Najim Ameziane[2], Johan P. De Winter[2]

[1]Division of Hematology, Department of Pediatrics, Gazi University Faculty of Medicine, Ankara, Turkey, and [2]Department of Clinical Genetics, VU University Medical Center, Amsterdam, the Netherlands
E-mail: drdagutf@ttmail.com and daslan@gazi.edu.tr
Received: 23 July 2014, Accepted: 31 October 2014

SUMMARY: Aslan D, Ameziane N, De Winter JP. Molecular diagnosis of Fanconi anemia with next-generation sequencing in a case with subtle signs and a negative chromosomal breakage test. Turk J Pediatr 2015; 57: 282-285.

Fanconi anemia (FA) is an inherited disorder characterized by malformations, marrow failure, and predisposition to cancer. Birth defects and laboratory features are characteristic and helpful in diagnosis, when present. Chromosome fragility is pathognomonic in the diagnosis. However, in some cases, there are no obvious physical anomalies or suggestive hematologic abnormalities, and inconclusive diagnostic tests have also been described. In such cases, a molecular diagnosis is required. This approach presents some advantages, especially in populations with a high incidence of FA and of consanguinity. Herein, we present a case with mild phenotypic features, inconclusive hematological findings and a negative breakage test. The diagnosis of FA was confirmed with next-generation sequencing. To our knowledge, this is the first publication of a FA patient being molecularly diagnosed utilizing this method since its introduction. Given its technical and financial features, we suggest that next-generation sequencing might be an alternative first-line diagnostic test for selected cases from particular populations.

Key words: Fanconi anemia, chromosomal breakage test, molecular diagnosis, next-generation sequencing.

Fanconi anemia (FA) is a rare genetic instability syndrome characterized by developmental defects, bone marrow failure and a high risk of cancer[1]. Clinical manifestations are diverse and variable. A large spectrum of hematological presentations, ranging from normal findings to severe aplastic anemia, may also be observed[2]. Therefore, arriving at an accurate diagnosis on the basis of clinical and hematological manifestations is difficult. Accompanying hemoglobinopathies may further complicate the diagnosis by modulating some of the suggestive hematological indices[3]. Complications of the disease are very serious (i.e., progressive pancytopenia, acute leukemia, aggressive solid tumors). A chromosomal breakage test, based on the extraordinarily sensitive response of FA cells to DNA cross-linking agents, is the gold standard for the ultimate diagnosis of FA[4]. In some cases, however, the breakage test is inconclusive (false negative or false positive), and molecular confirmation of the clinical diagnosis is required[5]. Confirmation of the FA diagnosis at the DNA level is important for many purposes, particularly for genetic counseling. Herein, we present a case with mild phenotypic features of FA, inconclusive hematological findings due to accompanying thalassemia and, most importantly, a negative chromosomal breakage test on repeated occasions. Diagnosis of FA in this patient was possible only after an exhaustive investigation; the pathogenic FA mutation was detected with next-generation sequencing.

Case Report

The subject of this report is a 28-month-old boy with thrombocytopenia. Thrombocytopenia had been detected incidentally, and thereafter, a

hematology consultation was ordered. He was born as the first child of a nonconsanguineous Turkish couple. His prenatal and natal history was unremarkable. He was healthy but had been underweight since birth. Evaluation for failure to gain weight revealed no pathology. In his infancy, the parents had consulted a plastic surgeon regarding a thumb anomaly, but no abnormality was determined. Physical examination revealed a normal height of 90 cm (percentile 50-75) and a weight of 10 kg (percentile 3-10). In evaluation of the skin, a subtle hypopigmented lesion on the nuchal area and a few hyperpigmented lesions on the left groin and upper thigh were observed. The left thumb was mildly hypoplastic compared to the right thumb (Fig. 1). Laboratory investigation on admission revealed hemoglobin: 7.9 g/dl, hematocrit: 24.2%, mean corpuscular volume: 71.4 fl, mean corpuscular hemoglobin: 23.5 pg, mean corpuscular hemoglobin concentration: 32.9 g/dl, erythrocyte count: 3.4 x 10^2/L, red cell distribution width: 21.8%, reticulocyte count: 0.9%, white blood cell count: 5.9 x 10^9/L (with an absolute neutrophil count of 1.4 x 10^9/L) and platelets: 85 x 10^9/L. Hemoglobin F level was 12.1%. Serum vitamin B_{12} and folate levels were within normal limits. Abdominopelvic ultrasonography findings were normal. Blood culture and serological tests to identify infectious pathogens that might cause thrombocytopenia were negative, and bone marrow aspirate, with a normal cellularity, excluded a malignant condition. Microcytosis could not be explained by iron deficiency (serum iron 87 μg/dl, serum iron binding capacity 306 μg/dl, transferrin saturation 29%, serum ferritin 188 μg/dl). Hemoglobin studies, conducted using high-performance liquid chromatography, revealed hemoglobin A_2 at 5%. The combination of reduced erythrocyte indices with normal body iron status and elevated hemoglobin A_2 level was consistent with beta thalassemia minor. Mutation analysis confirmed that he was heterozygous for the beta thalassemia mutation of IVS-I-110 (G/A). In view of the clinical and hematological features, a clinical diagnosis of FA was made. However, studies of chromosomal breakage induced by diepoxybutane (DEB), repeated on two separate occasions in two different laboratories, showed normal chromosomal breakage scores. Our attempts to provide a skin fibroblast culture for chromosomal breakage analysis to investigate somatic mosaicism failed due to technical issues. Despite these diagnostic difficulties, oxymetholone (1 mg/kg/day) was initiated to treat the persistent thrombocytopenia, which was considered to be the hematological manifestation of the underlying FA. The patient showed a good and sustained hematological response. The latest investigation at 34 months after initiation of treatment showed hemoglobin: 10.9 g/dl, mean corpuscular volume: 74 fl, reticulocytes: 2.7%, leukocytes: 6.6 x 10^9/L, neutrophils: 2.9 x 10^9/L and platelets: 150 x 10^9/L. To continue the investigation of FA, peripheral blood was obtained from the patient and the parents, after receipt of informed consent. DNA, isolated by standard protocols, was studied in the Department of Clinical Genetics, VU University Medical Center, Amsterdam, the Netherlands. A homozygous mutation of c.1615del;p.D539TfsX66 in the *FANCA* gene was found in the patient. Several polymorphisms in a homozygous state, confirming possible consanguinity in the family, were also detected. Genetic counseling was provided.

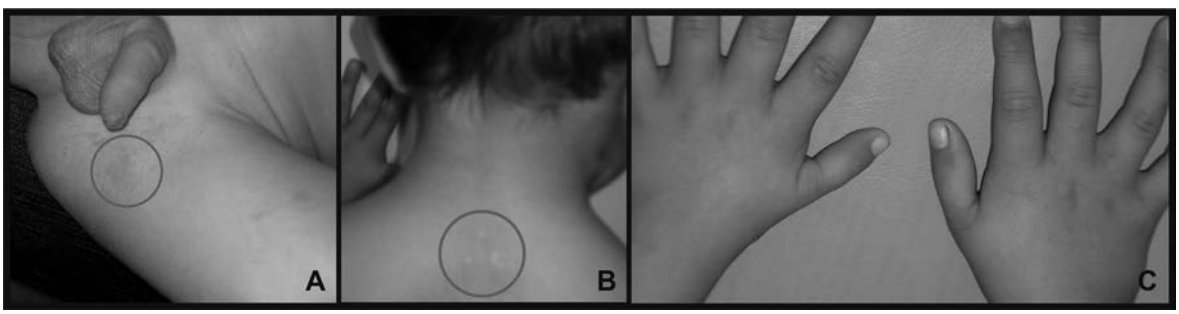

Fig. 1. Clinical features of the patient: Skin pigmentation abnormalities, including a hyperpigmented lesion on the left groin (A) and a subtle hypopigmented lesion on the nuchal area (B); Hypoplasia of the left thumb (C).

Discussion

In FA, congenital anomalies may affect any organ system. The most frequent somatic abnormalities include skin (i.e., hyperpigmentation and café au lait spots), skeletal (i.e., abnormal thumbs and radii, scoliosis), genitourinary (i.e., horseshoe kidney), gastrointestinal (i.e., duodenal atresia), cardiac and neurological anomalies[1,2]. As hematological abnormalities, patients with FA generally develop some degree of marrow dysfunction, ranging from mild asymptomatic cytopenia in any lineage (thrombocytopenia being the first) to severe aplastic anemia, myelodysplastic syndrome (MDS) or acute myeloid leukemia (AML). Most patients with FA have elevated hemoglobin F and macrocytosis[1,2]. However, approximately one-third of individuals with FA have no overt physical abnormalities[2]. FA patients with normal hematological findings have also been reported[2]. Thus, the absence of physical abnormalities or marrow failure findings does not exclude the diagnosis. In some populations, such as Mediterranean populations living in the thalassemia belt, the presence of accompanying hemoglobinopathies may also render the diagnosis difficult by modulating macrocytosis, one of the suggestive hematological findings[3], as seen in our patient. Alteration in the hemoglobin F level resulting from the underlying thalassemia may cause diagnostic confusion as well. There are also other syndromes with phenotypic features overlapping those of FA, such as dyskeratosis congenita and Nijmegen breakage syndrome[4,5]. For these reasons, it is important first *not to overlook* patients due to soft signs, and then *to properly diagnose* those patients with clinical findings.

The classic diagnostic test for FA is a chromosomal breakage test[6]. In this test, sensitivity of FA cells to the clastogenic effect of DEB and other DNA interstrand cross-linking agents is assessed. Abnormal response to cross-linkers provides a reliable cellular marker for the diagnosis. This test is not available in all laboratories, however. Furthermore, it can yield false negative results due to possible somatic mosaicism in some FA patients[4]. In addition, other genetic disorders with false positive breakage test results (such as Nijmegen breakage and Roberts syndromes) have been described[7]. In such conditions, the chromosomal breakage test is inconclusive, and confirmation of the FA diagnosis at the DNA level is crucial. Indeed, an increasing number of events bringing into question the diagnostic efficiency of the DEB test have come to light[8], and the occurrence of such events has led to arguments as to whether the use of this test in the diagnosis of FA should be reevaluated. In any case, molecular diagnosis of FA could present some advantages (e.g., it would make it possible to elucidate the genetic subtype of the patient for purposes of treatment and prognosis, carrier testing and preimplantation genetic diagnosis in future pregnancies, and to rule out FA in potential bone marrow transplantation donors who are phenotypically and hematologically normal). Among the current molecular methods, the most recently introduced—next-generation sequencing[9]—is of particular interest. This is a comprehensive mutation detection approach for FA, based on massive parallel sequencing. As compared to the conventional Sanger sequencing-based mutation screening approach, it is reported to be time-saving, inexpensive and capable of detecting some types of disease-causing aberrations that cannot be detected with Sanger sequencing[9]. Furthermore, it has been suggested that the obstructive effects presented by some pseudogenes during sequencing of genomic DNA by the Sanger method might be bypassed with this approach[9]. This novel method has already been double-checked against conventional Sanger sequencing for almost all genetic subtypes of FA (including the *FANCA* subtype detected in our patient), and all the FA mutations identified by Sanger sequencing were also detected with this method[9]. Therefore, the result in this particular patient was accepted as accurate, even though Sanger confirmation was not available due to financial issues. To our knowledge, this is the first publication of the molecular diagnosis of a Fanconi anemia patient provided firstly and solely by next-generation sequencing since the introduction of this method. Previous cases in whom next-generation sequencing was studied had been molecularly diagnosed using other methods and then re-diagnosed as a cross-check. In populations with a high rate of consanguinity, the frequency of autosomal recessive disorders such as FA is high, and

genetic counseling is important. Under such conditions, it would seem appropriate to choose a molecular diagnosis method that provides fast, reliable results at low cost. Given its technical and financial features, next-generation sequencing might meet these criteria.

In conclusion, due to inconclusive chromosomal breakage test results and other confounding factors, a definitive diagnosis was achieved in this patient only after prolonged and exhaustive investigation. The investigators overcame difficulties that could easily have led other clinicians to abandon the effort. Confirmation of the clinical diagnosis was possible using next-generation sequencing. It should certainly be noted that not every patient would benefit from such a prolonged approach. But we do believe that this particular case experience, albeit a single one, might contribute to the increasing arguments questioning the role of the DEB test in the diagnosis of FA, particularly in populations with a high incidence of FA and of consanguinity. We suggest that in the future the DEB test might be replaced by a molecular method, specifically next-generation sequencing, as the alternative first-line test in selected patients from vulnerable populations in whom FA is clinically suspected.

REFERENCES

1. Dokal I. Fanconi's anaemia and related bone marrow failure syndromes. Br Med Bull 2006; 77-78: 37-53.
2. Eiler ME, Frohnmayer D, Frohnmayer L, Larsen K, Owen J (eds). Fanconi Anemia: Guidelines for Diagnosis and Management (3rd ed). Eugene, OR: Fanconi Anemia Research Fund, Inc.; 2008.
3. Alter BP. Modulation of macrocytosis in aplastic anemia. Am J Hematol 1998; 57: 92.
4. Alter BP. Diagnosis, genetics, and management of inherited bone marrow failure syndromes. Hematology Am Soc Hematol Educ Program 2007: 29-39.
5. New HV, Cale CM, Tischkowitz M, et al. Nijmegen breakage syndrome diagnosed as Fanconi anaemia. Pediatr Blood Cancer 2005; 44: 494-499.
6. Auerbach AD. Fanconi anemia and its diagnosis. Mutat Res 2009; 668: 4-10.
7. Gallego MS, Barreiro CZ, Danielian S, Rosenweig SD. Positive diepoxybutane test in a patient with Nijmegen breakage syndrome. Pediatr Blood Cancer 2007; 49: 110.
8. Dokal I, Chase A, Morgan NV, et al. Positive diepoxybutane test in only one of two brothers found to be compound heterozygotes for Fanconi's anaemia complementation group C mutations. Br J Haematol 1996; 93: 813-816.
9. Ameziane N, Sie D, Dentro S, et al. Diagnosis of Fanconi anemia: mutation analysis by next-generation sequencing. Anemia 2012; 2012: 132856.