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Clinical Applicability of FANCD2 Mono-Ubiquitination Test for Fanconi Anemia Diagnosis and a Suggestion for an Algorithm

[Fanconi Anemisi Tanısında FANCD2 Mono-Ubikitinasyon Testinin Klinik Uygulanabilirliği ve bir Algoritma Önerisi]

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ABSTRACT

Objectives: Fanconi anemia is caused by mutations in related FANC genes that are involved in DNA repair pathways. Diepoxybutane induced chromosome break test in lymphocytes if positive is affirmative for the diagnosis. However, in 20-25% of the cases the test results have intermediate values which are mostly attributed to somatic mosaicism and cause an ambiguity for diagnosis. Here, we utilize the relatively new and rarely used FANCD2 mono-ubiquitination test to facilitate and resolve the diagnostic problem in a clinical scheme.

Methods: Fifteen patients with different diepoxybutane test results (positive, intermediate, negative) and four control subjects were included to the study. Western blot analyses in cultured peripheral blood lymphocyte-isolates were done with a specific antibody to discriminate between ubiquitinated and non-ubiquitinated forms of FANCD2.

Results: The test confirmed the diagnosis in one of the diepoxybutane positive patients. In four cases out of seven with intermediate test values we were able to make the right diagnoses. For the other three in this group, FANCD2 test neither confirmed nor rejected the diagnoses which may be due to high somatic mosaicism or due to a defect in the downstream part of the Fanconi pathway. The ubiquitinated isoform of FANCD2 were detected in all six diepoxybutane negative cases.

Conclusion: It is expected that adopting FANCD2 mono-ubiquitination Western blot analysis as a diagnostic tool with the proposed algorithm could facilitate the management of Fanconi Anemia without compromise.

Keywords: Fanconi anemia, FANCD2, mono-ubiquitination, diagnosis, DEB testing algorithm

ÖZET

Amaç: Fanconi anemisi DNA onarım yolaklarında rolü olan *FANC* genlerindeki mutasyonların neden olduğu bir hastalıktır. Diepoksibütan ile uyarılmış lenfosit kırık testinin pozitifliği tanı koydurucudur. Ancak olguların %20-25'inde sonuçlar ara değerlerde, ender de olsa negatif saptanabilirler ve bu durum çoğunlukla somatik mosaisizm ile açıklanır. Bu çalışmadaki amacımız, klinik uygulama düzeninde ülkemizde henüz uygulanmayan FANCD2 mono-ubikitinasyon testinin tanı için standart kabul edilen kromozom kırık testi ile birlikte kullanımı ile tanısal sorunlara kolaylaştırıcı çözüm sağlama potansiyelini sınamaktır.

Gereç ve Yöntemler: Bu çalışmada farklı diepoksibütan test sonuçlarına göre (pozitif, ara değer, negatif) seçilmiş 15 olguda izole lenfosit kültürlerinden elde edilen hücre lizatlarında FANCD2 antikoru ile Western blot incelemesi gerçekleştirilerek bu proteinin ubikitinli ve ubikitinsiz izoformlarının ayrımı sağlanmıştır.

Bulgular: Test ile DEB pozitif bir olguda tanı doğrulanmış, aradeğerli olguların dördünde Fanconi tanısı konularak olgu izleminin sürüncemede kalması engellenmiştir. Ara değerli üç olguda FANCD2 testi tanı açısından belirleyici olmamıştır. Bu durum hastaların lenfosit popülasyonlarındaki yüksek somatik mozaiklik veya ilgili yolağın alt kısımlarındaki bozukluklar ile açıklanmaktadır. DEB negatif olguların hiçbirinde FANCD2'nin mono-ubikitine izoformu saptanmamıştır.

Sonuçlar: Western blot yöntemi ile FANCD2 mono-ubikitinasyon analizinin, önerilen algoritma eşliğinde uygulanması ile Fanconi anemisinin tanısal yaklaşımına ödünsüz şekilde kolaylık sağlaması beklenmektedir.

Anahtar sözcükler: Fanconi anemisi, FANCD2, mono-ubikitinasyon, tanı, DEB testi, algoritma

Introduction

Fanconi anemia (FA) is a chromosome instability syndrome caused by cross-linking agents and ionizing radiation in susceptible cells (1,2). FA is rare disease with low prevalence and incidence and has an estimated heterozygous carrier frequency of 1 in 300, although this may be higher in certain ethnic groups (3,4). FA is inherited in autosomal recessive or in X-linked fashion depending on the chromosomal loci of responsible genes. The spectrum and frequency of clinical manifestations are well documented (5,6). Sensitivity to DNA cross-linking agents like mitomycin C and diepoxybutane (DEB) is a well known cellular response in this disorder (7). For most of the FA cases the clinical diagnosis with ascertainment is limited due to a large number of variant or sometimes subtle clinical manifestations that may arise relatively late in childhood. Pancytopenia alone, or with growth retardation, or with radius anomalies are conspicuous findings in FA (8). Most of the time hyperpigmentation is also associated with these clinical manifestations. However, absence of one or more of these frequent characteristics of this disease may hinder correct or timely diagnosis (9,10). A prompt diagnosis of this condition postpartum or prenatally is crucial for monitoring the patient's prognosis, and for implementing a suitable treatment protocol. Bone marrow transplantation is often the only choice in the presence of aplastic anemia or hematological malignancy and would require the selection of proper donor from the family with the exclusion of FA.

FA proteins are involved in DNA repair and constitute a pathway interacting with other proteins during DNA replication and repair (3,12). Currently there are thirteen genes identified and with their respective protein products they constitute the complementation groups which are responsible for FA disorder and these proteins with a "FANC" or "FA" prefixes often called as subtypes A, B, C, D1/BRCA2, D2, E, F, G, I, J, L, M and, N were elaborately analyzed in patients' cell lines to form functional complementation partners by somatic hybridization studies (11). Eight of these proteins, (FANCA, FANCB, FANCC, FANCE, FANF, FANCG, FANCL and FANCM) form the FA core complex and move into the nucleus through nuclear localization signals which some members of this complex bare. In response to DNA-damage during replication this complex activates FANCL, an E3 ubiquitin ligase, which facilitates the monoubiquitation of FANCD2 and the FANCD2 associated protein FANCI. FANCI were found to enhance repair although its role is not as crucial as FANCD2 ubiquitination (12). FANCD2, upon mono-ubiquitination interacts with other members of FA proteins (FANCD1/BRCA2, FANCJ, FANCN) as well as with other DNA-repair proteins (BRCA1, RAD51) which take place in DNA repair and chromosomal stability (12) . The pathway might be regarded as two but consecutive events, the formation of core complex which results in FANCD2 mono-ubiquitination - a prerequisite for FA/BRCA pathway related DNA repair action - and the downstream events with the contribution of other FA proteins and their associates. Most of the patients have mutations in one of the FA genes that encode the core complex. The majority of the mutations are seen in FANCA (66%) followed by FANCC (10%), FANCG (9%). The prevalence of FANCB , FANCD1, FANCD2, FANCF, FANCI and FANCJ is approximately 2% each (12). Thus, the sum of the mutations seen in the genes of core-complex related proteins explains the molecular defect in more than 90% of FA cases. Shimamura et al.(13) have deveolped a test based on immunoblotting of FANCD2 to detect mono-ubiquitinated and non-ubiquitinated isoforms of this key protein. The diepoxybutane (DEB) test which is based on the induction of cellular DNA to crosslinking in cultured cells causing breaks and abnormal structures in the chromosomes during metaphase is a phenotypic feature and currently the preferred diagnostic test in FA. Molecular genetic testing allows the detection of mutated gene in more than 90% of the cases but, they are expensive, time-consuming and can be troublesome due to genetic heterogeneity (3). Complementation analyses usually performed for research purposes are not suitable for routine diagnostic testing. Since 1989, according to the IFAR guidelines the positive DEB test alone is considered to be sufficient for FA diagnosis and average chromosomal breakage frequency is used as a criteria for DEB positiveness, hence not all examined cells should contain at least one break (14). The average chromosomal breakage frequency in peripheral blood lymphocytes is 0 to 0.05 breaks per cell in normal individuals and 0.02 to 0.85 per cell in FA patients. The DEB-induced chromosomal breakage frequency ranges from 0.00-0.10 breaks per cell for normal individuals and for the FA patients from 1.00-23.9 breaks per cell. However, in some patients the percent of cells exhibiting chromosomal breakage from DEB-cultures can vary from as low as 10% to as high as 100% (15). Such results fall between a range that hinders acertainment of FA through DEB testing. These instances are suggested to arise from somatic mosaicism that can be seen in hematopoietic cells where two population of cells differ in their sensitivity to clastogenic agents hence, resulting in the disease phenotype reverting back to normal in the relevant cell clones and account for 10-25% of the cases (16,17).

DEB testing is repeated as a routine approach in FA diagnosis for these cases. While a positive test result may warrant FA diagnosis in retesting, the problem is not resolved for most of the cases. In the presence of a prediagnosis based on strong clinical evidence an intermediate or even a negative DEB result complicates the management of the disease (15,16). In the current study we review and adopt the FANCD2 mono-ubiquitination by Western blot analysis for a number of cases and subjects for diagnosis and control. State of the art techniques cannot be used in routine diagnostic methods mainly due to

multigenic and multiallelic nature of FA. The advantage of FANCD2 monoubiquitination test arises especially for its discrimination propertry between disease and non disease status for 90% of the cases, the method itself was easy in application and for implementation as part of a routine testing. Besides its high specificity the results obtained were decisive especially for some cases where DEB tests did not accord with strong clinical evidence for FA.

Materials and Methods

Cases:

Fifteen patients referred to the Departments of Medical Genetics or Pediatric Hematology of Ankara University Faculty of Medicine on the basis of clinical manifestations and previous DEB testings and four healthy volunteers as controls were included to the study for FANCD2 mono-ubiquitination test between December 2009 and March 2010. Two of the patients were previously diagnosed as FA with positive DEB testing results. Seven patients had intermediate chromosome break ratios (>%10) therefore baring uncertainty for FA diagnosis with DEB testing. The rest of the patients and subjects were either consulted to exclude FA or to confirm the absence of FA disease thus they can be eligible for bone marrow donorship for their kins. Informed consents were obtained from all of the subjects or from their parents and/or guardians. The study was approved by the Ethical Committee for Clinical Researches of Ankara (02.12.2009/05-57).

Lymphocyte cultures and preparation of cell lysates:

Peripheral blood samples were layered on to Histopaque-1077 (Sigma Aldrich, USA) in equal volumes and mononuclear cells were seperated by density gradient centrifugation at 400xg for 30 min. at room temperature. Buffy coats were collected, transferred to fresh tubes, washed and centrifuged three times at 250xg for 10 min. The pellets were resuspended in equal volume of RPMI 1640 (PAA Laboratories GmbH) and cells were counted in a hemocytometer. Lymphocytes were seeded ($5x10^5$ cells/ml) in a final volume of 5 ml RPMI 1640 culture medium supplemented with 15% heat-inactivated fetal bovine serum (Biochrom AG), L-glutamine (1mM), streptomycin (100 mg/ml), penicillin (10 U/ml), phytohemaglutinin (10 mg/ml;_Biological Industries Israel Beit-Haemek) as mitogen, and incubated for a period of 72 h at 37°C. Cultures were harvested, resuspended in PBS and counted.

Cytogenetic analysis of chromosome breaks:

Hypersensitivity to clastogenic effect was tested with DNA cross-linking agent DEB in the new patients as part of a routine analysis. Heparinized peripheral blood samples (0.5 ml) were obtained and cultured in replicates with conditions described in the previous section. DEB was added to one set of cultures at a final concentration of 0.1 mg/ml after 24 h, the other set remained untreated. Slides were Giemsa stained and 50 metaphases were inspected and scored for chromosome breakage. In accord with the International Fanconi Anemia Registry criteria (7), patients were diagnosed as having FA when the frequency of breaks/cell in the metaphases from treated cultures was over 1.00. Any ratio over 0.1 but less than 1.00 were considered as intermediate values.

Western blot analysis:

Lysis buffer (50 mM Tris-HCl pH 8, 1% Nonidet-P40, 150 mM NaCl, and mix protease inhibitors) were added to $5x10^6$ cells harvested from each culture to yield approximately 50-100 ug of total protein load for a final concentration in sample buffer, determined with Bradford reagent (BioRad) spectrophotometric protein quantitation assay, incubated on ice for 15 min, centrifuged down and stored at -20°C as cell lysates. Samples were loaded to 5% stacking, 7% seperating sodium dodecyl sulfate (SDS) polyacrylamide gels in sample buffer (50 mM Tris-HCl, 10% 2-mercaptoethanol, 2% SDS, bromophenol blue) preheated to 97°C for 3 min and were run along with protein molecular weight markers (Sigma Aldrich) for 15 min under 10 mA and 45 min under 20 mA constant currents for stacking and seperating parts respectively. Proteins were then transferred to polyvinylidene fluoride (Immobilon –P, Millipore) membranes prewet with absolute methanol and 1X transfer buffer (25 mM Tris base, 200 mM glycine) in a transfer apparatus containing 1X transfer buffer. The membranes were blocked with 5% drymilk in TBS-T buffer (50 mM Tris-HCl, 150 mM NaCl, 0.1% Tween 20), incubated first with rabbit anti-human FANCD2 polyclona*l* primary antibody (Ab2; Calbiochem/Immunochemicals, USA) for 2 h at 1:1000 dilution and second with goat anti-rabbit antibody conjugated to horseradish peroxidase at 1:10000 diliution. Membranes were visualized by enhanced chemiluminescence method using Luminal Reagent (Santa Cruz Biotechnology, USA) according to the manufacturers inscriptions. After being exposed to X-ray films blots were inspected for the presence or absence of the large bands coresponding to the mono-ubiquitinated (162 kDa) or small non-ubiquitinated (155 kDa) forms of FANCD2 (FANCD2-L/S). Digital images were produced with a computarized CCD camera system.

Results

Western blots of mono-ubiquitinated FANCD2 protein with a specific antibody in cultured blood mononuclear cell lysates of four healthy controls all show one large (162 kDa) and one small (155 kDa) band corresponding to mono-ubiquitinated and non-ubiquitinated protein isoforms respectively. In Figure 1 single and double banded patterns from various samples are distinguishable. The demograhics of each case, their clinical findings, conventional cytogenetic analyses results, and chromo-

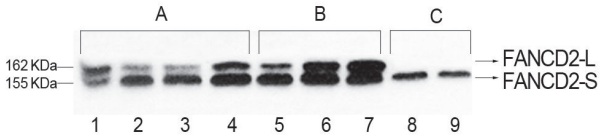

Figure 1. A representative image of Western blot analysis of FANCD2 proteins. Cell lysates of mono-nuclear isolates were seperated in SDS polyacrylamide gel electrophoresis, transferred to membranes, incubated with *rabbit anti-human FANCD2 polyclonal* primary antibody then with horseradish peroxidase conjugated secondary antibody, and visualized by enhanced chemiluminescence. Upper and lower bands represent mono-ubiquitinated and non-ubiquitinated forms of FANCD2 respectively (FANCD2-L/S). (A) Lanes 1-4: control subjects. (B) Lanes 5-6: DEB negative cases (#14, #15), lane 7: a DEB intermediate case (#7). (C) Lane 8: DEB intermediate case (#5), lane 9: DEB positive case (#1). Left most lane was alocated for molecular weight standarts in the gel and is not visible in the blot.

some break ratios are provided in Table 1. The patients were divided into three subgroups in accordance with their DEB results and presented in Table 2 with Western blot images. The first two cases denoted with a "+" sign were FA patients and their diagnoses had been confirmed with positive DEB results. The second group (cases 3-9) with uncertain DEB results (?) due to inadequate percentage of chromosome breaks were referred with FA prediagnosis. The last group (cases 10-15) who were either referred for exclusion or for confirmation of absence of FA were all tested DEB negative.

In one of the two cases with unequivocal DEB positive results FA diagnosis was confirmed with the absence of FANCD2-L (case #1), the other case noted as FANCD2-L/S positive, thus mono-ubiquitination test

Table 1. Clinical Synopsis of the Study Cases

Case #	Age	Sex	Clinical phenotype	Family History	Related to	FANC Mutation Analysis
1	13	M	thumb abnormality, hyperpigmentation	No	case #8	No
2	6	F	aplastic anemia, growth retardation, microcephaly, café au lait spots	Parents: distant relatives	No	No
3	7	M	cryptorchidism, left renal hypoplasia thrombocytopenia, pes equinovarus	Aunt (father side) with FA died at 17	No	No
4	6	F	growth retardation, microcephaly, dysplastic auricula, bilateral thumb abnormality, café au lait spots aplastic anemia (Dx: at age 5)	Parents: distant relatives	No	No
5	6	F	growth retardation, micrognathia, short neck, café au lait spots bicytopenia, microcytic anemia	Parents: 1st cousins	No	No
6	12	M	bicytopenia, hyperpigmentation, café au lait spots	Parents: 1st cousins 4yearold brother: growth retardation 2nd cousin: suspected FA	No	No
7	13	F	wilms tumor (Dx: at age 2) no other findings acute myelogenous leukemia (Dx: at age 11)	Parents: 2nd cousins 15yearold brother: Growth retardation	No	BRCA2 c.9052 9057 del6
8	4	M	healthy considered as a bobe marrow donor	Brother: DEB tested positive, (Dx: FA at age10)	case #1	No
9	1.5	F	short stature, low weight, microcephaly, dysplastic auricula, pectus excavatum bilateral thumb abnormalities, low red cell count	Parents: 2nd cousins	No	No
10	12	M	Hyperpigmentation, hypertrichosis bilateral thumb abnormalities, aplastic anemia	Parents: 1st cousins	No	No
11	16.5	M	acute myelogenous leukemia (Dx: at age 16)	No	No	No
12	17	M	acute rheumatoid arthritis (Dx: at age 1.5) hearing loss (otitis media complication) wide spread vitiligo, café au lait spot pancytopenia (Dx: at age 12)	Parents: 1st cousins Younger brother: Died at 13 from pancytopenia	No	No
13	5	F	healthy bone marrow donor candidate	Parents: 2nd cousins Younger sister: FA (Dx: at age 2), she's not a subject of the current study	case #14	No
14	10	F	healthy bone marrow donor candidate	Same as above	case #13	No
15	15	F	dyserythropoietic anemia (Dx: at age 5) cardiomyopathy	Parents: 1st cousins Younger brother: Died at 6 months, cause unknown	No	No

Table 2. Western blot and chromosome break results of 15 Fanconi anemia cases

Case#	Spontenous breaks/cell (%)	DEB breaks/cell (%)	DEB test confirmed diagnosis	FANCD2 Western blot	FANCD2-S/ FANCD2-L
1	16	100	+		+/-
2	4	182	+		+/+
3	40	36	?		+/-
4	26	22	?		+/-
5	46	34	?		+/-
6	37	38	?		+/-
7	10	23.6	?		+/+
8	10	34	?		+/+
9	10	12	?		+/+
10	10	10	_		+/+
11	8	4	_		+/+
12	0	8	_		+/+
13	2	4	_		+/+
14	0	0	_		+/+
15	0	6	_		+/+

failed to correlate with FA diagnosis. The absence of mono-ubiquitination was consistent with FA diagnosis in five out of nine cases (cases 1-9) whose chromosome break ratios were either positive or intermediate. In cases from 3 to 6 FANCD2-L were lacking. The absence of monoubiquitinated FANCD2 protein is a confirmation of FA. Case #7 was previously analyzed for possible *FANC* functional mutations to reveal maternally inherited deletion in one copy of *BRCA2* gene. Otherwise healthy, case #8, the younger brother of DEB positive case #1 was tested to have an intermediate DEB induced break ratio (34%) in the medical genetics department two years ago. In the Western blot analysis he appeared to have FANCD2-L as normal cases do. On the contrary case #9, whose DEB test results fall into intermediate range had a highly convincing clinical phenotype in favor of FA. For these three cases (#7-#9) with intermediate chromosome break ratios, FA diagnosis could not be ruled out since they all had the monoubiquitinated isoform of FNACD2. The members of the last group with DEB test negative samples did show both bands corresponding to the mono-ubiquitinated (FANCD2-L) and non-ubiquitinated FANCD2 (FANCD2-S) isoforms.

Discussion

In the current study fifteen cases were screened for the presence or absence of mono-ubiquitinated form of FANCD2 with Western blot analysis. The cases were intentionally selected and included to the study depending on their DEB analyses results and reasons for their referral. Hence we included two cases to the study with strong clinical evidences and following DEB positiveness. In the first patient who was being followed with typical clinical features of FA for four years, we confirmed the diagnosis with the FANCD2 mono-ubiquitination testing. Likewise, the second patient who displayed a profound FA phenotype both for DEB-induced chromosome breaks and for clinical manifestations did not show a defect in the formation of FA core-complex. It's known that 10% of the patients have a defect beyond this point (18). This result indicated to a fault in the downstream events responsible for proper FA pathway function for this patient. Nevertheless, the positive DEB test alone was affirmative for the diagnosis. The following seven cases all showed intermediate chromosome breaks in DEB tests. This was a constraint for a decisive therapeutic action from clinical point of view. Four of these cases (#3-6) with supporting clinical evidence for the presence FA were in a situation such as mentioned. In several studies (19-23), intermediate DEB results in FA are attributed to somatic mosaicism due to reverse mutations taking place in hematopoietic cells and causing to the existance of two different populations of lymphocytes and their opposite reactions to DNA cross-linking agents while non-hematopoietic cells consistently show sensitivity against DEB and similar agents. This, in fact, suggested the use of alternative tissues for DEB testing, such as fibroblasts. In contrast to peripheral blood cells, tests performed with solid tissue cells are invasive on patients, take longer time and difficult to cultivate. Here, the absence of FANCD2 mono-ubiquitination in all these four cases indicated to core-complex formation problems and their diagnosis as FA were undisputed therefore further testing were avoided. The other three cases (#7-9) also showing intermediate DEB results were tested positive for the FANCD2-L, so both tests were non-confirmative for the diagnosis. Displaying a characteristic clinical FA phenotype, case #7 has been previously investigated for the frequent *FANC* mutation loci and reported to have maternally inherited BRCA2_c.9052_9057 del6 mutation in the heterozygote form. Further investigations are needed, would this mutation is related to the pathogenesis in FA since the patient bears a single copy of the mutation. However, BRCA2 protein has a role in the downstream part of FA pathway and its biallelic mutations are known to disrupt FA repair function. (24) Case# 8 was referred to us being considered as a bone marrow donor candidate for his brother (case#1) who was tested DEB positive with FA findings. His clinical examinations revealed no signs of a disorder, but his DEB test result turned out to be in the intermediate range. Case#

9, on the other hand, who had many phenotypic findings related to disease spectrum was not confirmed to have FA due to her DEB result close to lower limit value (12% breaks/cell). FANCD2 mono-ubiquitination test neither confirmed nor rejected FA diagnosis in cases 8 and 9. Although for different reasons their situations demand a clarification. For case# 8 it is possible that the hematological manifestations may yet to emerge regarding his age. And, his intermediate DEB result (34% breaks/cell) may suggest a somatic mosaicism in the lymphocyte population. In the later case, the presence of a severe phenotype whether caused by FA or not should be further investigated for a prompt and right diagnosis. In either case, the presence of two isoforms of FANCD2 means the eight partners of FA core-complex are normal along with FANCD2 itself. Considering a contingency for FA, if further DEB tests would not help diagnosis, same procedure should be implemented in fibroblasts, else the molecular pathology should be investigated for genes functioning below core-complex (FANCD1/BRCA2, FANCJ, FANCN) by mutation analysiAll of the six cases from the DEB test negative group (#10-15) were detected positive for the ubiquitinated isoform of FANCD2. Although neither of the tests supported the diagnoses, each case should be approached and re-examined delicately for a final decision to exclude FA. For example, in case# 10, clinical findings cause a strong suspicion of FA with the presence of aplastic anemia, bilateral thumb anomalies, skeletal findings and hyperpigmentations. Besides, tri-radial figures noted in non-induced chromosome analysis with DEB negativity may be an indication of high somatic mosaicsm or a defect in the downstream of the pathway. Case# 11, with atypical age of onset of AML without any additional manifestations, case# 12, having lost a brother at 13 from pancytopenia and himself previously diagnosed with pancytopenia and bearing other clinical findings, were also referred to us for DEB test. The contribution of both tests was inconclusive and lab studies should be advanced as discussed previously. The next two cases (#13, #14) in the DEB negative group were sisters. they were being investigated as bone marrow donor candidates for their younger sisters with FA. They were tested negative in DEB and positive in FANCD2 mono-ubiquitination as expected. Their results were consistent with DEB test and FA exclusion was plausible with their clinical status considered. Case# 15 was referred for FA discrimination and mono-ubiquitinated FANCD2 was also found positive in this. Her clinical type was vaguely in favor of FA therefore investigating the etiology underlying the dyserythropoietic anemia could be more effective before pursuing other tests for FA.

After the cloning of FANCD2 gene the function of FA core-complex and its relation to FA pathway were better understood (25). Several research groups have focused on the formation of this complex and with its counterparts in detail. Garcia-Higuera et al. (26) showed that FANCD2 mono-ubiquitination is a prerequisite for its activation and the formation of the core-complex and for the FA pathway related events to advance. This group also was first to suggest the anti-FANCD2 immunoblotting to screen FA patients for upstream defects in FA pathway as a practical alternative to the currently used chromosome breakage test DEB or MMC. Shimamura et al. (13) has employed this test in a group of FA patients and they have provided the right diagnosis in four of the patients whose DEB test results were in intermediate–value zone due to somatic mosaicism. They have also come to conclusion that 90% of all FA gene mutations were responsible for the failure of FANCD2-L mediated formation of core-complex hence the result in FA disease phenotype. In another study 53 patients with a strong clinical support for FA prediagnosis were investigated by DEB and FANCD2 mono-ubiquitination testing in lymphocytes and fibroblast cultures (16) where 45 of the cases with DEB positive test results were confirmed to have FA with the absence of FANCD2 mono-ubiquitination in Western blot analysis. In six of these patients it was by means of FANCD2 testing in fibroblast- culture derived cells that this group was able to clarify the intermediate DEB results in patients were due to the presence of revertant lymphocyte population. For two of the cases neither test were useful for diagnosis, however an overall contribution of FANCD2 test was an impressive 96%. Therefore early diagnosis could be made possible even in the presence of somatic mosaicism with the utilization of the FANCD2 mono-ubiquitination test.

Following these pioneering studies relatively larger patient groups were studied for FANCD2 mono-ubiquitination along with the determination of complementation groups and related mutations of FANC genes (27). In 2009 Pilonetto et al.(28) reported the results of their studies in 87 patients which revealed 94% sensitivity and 100% specificity for the diagnosis of FA. The reliability of the test is also supported by the fact that while the absence of ubiquitinated form of FANCD2 is specific for FA, the chromosome breaks that are detected spontaneously or with response to cross-linking agents may also be seen in other chromosomal break diseases, such as Nijmegen syndrome (29).

The low frequency of the disease hindered us from using a statistical testing nor providing a contingency table to calculate the specificity and sensitivity of the method. However, the results of this current study - without any FANCD2 positives in DEB negatives - show high negative predictive value for FANCD2 testing. Due to the fact that 90% of the patients have mutations for the genes in upstream portion of the FA pathway, theoretically sensitivity of the the test should converge to 90%. Current data with only two DEB+ results is far from healthy evaluation. On the other hand, the data represent 100% specificity, with all the DEB- results coinciding with FANCD2 – results (Table 3).

Table 3. Cross tabulation of the Fanconi Anemia Test Results

Tests	DEB +	DEB-int.	DEB -	Total
FANCD2 +	1	4	0	5
FANCD2 -	1	3	6	10
Total	2	7	6	15

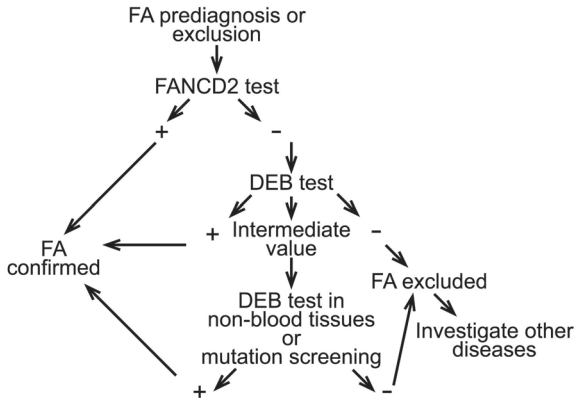

Figure 2. An algorithm proposed for the management of Fanconi anemia testing and diagnosis.

Despite to modest number of observations, tests that were lacking the FANCD2 mono-ubiquitination helped resolving the ambiguity in the FA diagnosis with certainty. Here, we provide an algorithm to be considered for the management of Fanconi anemia diagnosis (Figure 2). According to this algorithm first the FANCD2 mono-ubiquitination is tested for patients with prediagnosis and for exclusion of FA. A positive test result always warrants the presence of FA. Detection of FANCD2-L, a negative result, requires DEB testing. Positive DEB test confirms the diagnosis but for intermediate values DEB test should be repeated with the use of a different type of tissue other than blood. If a negative result is encountered in the non-blood tissue cells the diagnosis should be reconsidered. Where the clinical status is persistently in favor of FA, mutation analysis is the last resort for diagnosis. In conclusion, this study provided convincing results for adoption of FANCD2 testing in a clinical schema as part of the routine analysis together with chromosome break test in FA diagnosis.

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References

[1] Lobitz S, Velleuer E. (2006) Guido Fanconi (1892–1979): a jack of all trades. Nat Rev Cancer. 6: 893-8.

[2] Taylor AMR. (2001) Chromosome instability syndromes. Best Practice & Research Clinical Haematology. 14: 631-644.

[3] Auerbach AD. (2009) Fanconi anemia and its diagnosis. Mutat Res. 668: 4-10.

[4] BouchlakaC, Abdelhak S, Amouri A, Ben Abid H, Hadiji S, *et al.* (2003) Fanconi anemia in Tunisia: high prevalence of group A and identification of new FANCA mutations. J Hum Genet. 48:352–361

[5] Altay C, Alikasifoglu M, Kara A, Tuncbilek E, Ozbek N *et al.* (1997) Analysis of 65 Turkish patients with congenital aplastic anemia (Fanconi anemia and non-Fanconi anemia): Hacettepe experience. Clin Genet. 51:296–302.

[6] De Kerviler E, Guermazi A, Zagdanski AM, Gluckman E, Frija J. (2000) The clinical and radiological features of Fanconi's anaemia. Clin Radiol 55:340-5.

[7] Alter BP. (2008) Diagnostic Evaluation of FA. Fanconi anemia guidelines for diagnosis and management (Editors: Eiler ME, Frohnmayer D, Frohnmayer L, Larsen K, Owen J), s.33-39. Fanconi Anemia Research Fund, Inc.

[8] Butturini A, Gale RP, Verlander PC, Adler-Brecher B, Gillio AP, *et al.* (1994) Hematologic abnormalities in Fanconi anemia: an International Fanconi Anemia Registry study. Blood. 84:1650-5.

[9] Giampietro PF, Verlander PC, Davis JG, Auerbach AD. (1997) Diagnosis of Fanconi anemia in patients without congenital malformations: an International Fanconi Anemia Registry study. Am J Med Genet. 68:58-61.

[10] Liu JM, Auerbach AD, Young NS. (1991) Fanconi anemia presenting unexpectedly in an adult kindred with no dysmorphic features. Am J Med. 91:555-7.

[11] Ameziane N, Errami A, Léveillé F, Fontaine C, de Vries Y *et al.* (2008*) Genetic subtyping of Fanconi anemia by comprehensive mutation screening.* Hum Mut. 29;1:159-166.

[12] Moldovan GL, D'Andrea AD. (2009) How the Fanconi anemia pathway guards the genome. Annu Rev Genet. 43: 223–49.

[13] Shimamura A, Montes de OR, Svenson JL, Haining N, Moreau LA, *et al.* (2002) A novel diagnostic screen for defects in the Fanconi anemia pathway. Blood. 100:4649-4654.

[14] Auerbach AD, Rogatko A, Schroeder-Kurth TM. (1989) International Fanconi Anemia Registry: relation of clinical symptoms to diepoxybutane sensitivity. Blood. 73: 391-396.

[15] Auerbach AD. (2003) Diagnosis of Fanconi Anemia by Diepoxybutane Analysis. Curr Protoc Hum Genet. (Editors: Dracopoli NC, Haines JL, Korf BR, Moir DT, Morton CC, Seidman CE, Seidman JG, Smith DR), Jul; Chapter 8: Unit 8.7.1-8.7.15. John Wiley & Sons, Hoboken, NJ.

[16] Soulier J, Leblanc T, Larghero J, Dastot H, Shimamura A, *et al.* (2005) Detection of somatic mosaicism and classification of Fanconi anemia patients by analysis of the FA/BRCA pathway. Blood. 105: 1329-1336.

[17] Tischkowitz MD, Hodgson SV. (2003) Fanconi anaemia J. Med. Genet. 40;1-10.

[18] Taniguchi T, D'Andrea AD. (2006) Molecular pathogenesis of Fanconi anemia: recent progress. Blood. 107: 4223-4233.

[19] Jonkman MF. (1999) Revertant mosaicism in human genetic disorders. Am J Med Genet. 85: 361-4.

[20] Waisfisz Q, Morgan NV, Savino M, de Winter JP, van Berkel CG, *et al.* (1999) Spontaneous functional correction of homozygous Fanconi anaemia alleles reveals novel mechanistic basis for reverse mosaicism. Nat Genet. 22: 379-83.

[21] Youssoufian H, Pyeritz RE. (2002) Mechanisms and consequences of somatic mosaicism in humans. Nat Rev Genet. 3: 748-58.

[22] Hirschhorn R. (2003) In vivo reversion to normal of inherited mutations in humans. J Med Genet. 40: 721-8.

[23] Gregory JJ Jr, Wagner JE, Verlander PC, Levran O, Batish SD, *et al.* (2001) Somatic mosaicism in Fanconi anemia: evidence

of genotypic reversion in lymphohematopoietic stem cells. Proc Natl Acad Sci USA. 98: 2532-7.

[24] Howlett NG, Taniguchi T, Olson S, Cox B, Waisfisz Q, *et al.* (2002) Biallelic inactivation of BRCA2 in Fanconi anemia. Science 297: 606-609.

[25] Timmers C, Taniguchi T, Hejna J, Reifsteck C, Lucas L, Bruun D, et al. (2001) Positional cloning of a novel Fanconi anemia gene, FANCD2. Mol Cell. 7: 241-248.

[26] Garcia-Higuera I, Taniguchi T, Ganesan S, Meyn M S, Timmers C, *et al.* (2001) Interaction of the Fanconi Anemia Proteins and BRCA1 in a Common Pathway. Molecular Cell. 7: 249–262.

Nakanishi K, Moran A, Hays T, *et al.* (2001) Functional analysis of patient-derived mutations in the Fanconi anemia gene, FANCG/XRCC9. Exp Hematol. 29:842-849.

[27] Pinto FO, Leblanc T, Chamousset D, Le Roux G, Brethon B, *et al.* (2009) Diagnosis of Fanconi anemia in patients with bone marrow failure. Haematologica. 94(4): 487-95.

[28] Pilonetto DV, Pereira NF, Bitencourt MA, Magdalena NIR, Vieira1 ER, *et al.* (2009) FANCD2 Western blot as a diagnostic tool for Brazillian patients with Fanconi anemia. Braz J Med Biol Res. 42: 237-43.

[29] Antoccia A, Kobayashi J, Tauchi H, Matsuura S, Komatsu K. (2006) Nijmegen breakage syndrome and functions of the responsible protein, NBS1. Genome Dyn. 2006; 1:191-205.