J Korean Med Sci 1998; 13: 623-8
ISSN 1011-8934

Fanconi Anemia Screening by Diepoxybutane and Mitomicin C Tests in Korean Children with Bone Marrow Failure Syndromes

Fanconi anemia (FA) is an autosomal recessive disorder of progressive bone marrow failure in patients with congenital malformations. FA is different from acquired aplastic anemia (AA) in terms of the natural course and treatment options. As the frequency of FA is unknown in Korea, we conducted screening tests using DNA clastogenic agents, diepoxybutane (DEB) and mitomicin C (MMC) in southwestern Korea. Forty-three children with AA or other bone marrow failure syndromes and siblings of known FA were evaluated. Six patients with AA (6/24=25.0%) and a 2-month-old patient with myelodysplastic syndrome were found to have increased chromosomal breakage to both DEB and MMC, confirming the diagnosis of FA. No overlap in chromosomal breakage to both agents was found between the FA group and non-FA group. The frequency of FA in this study, much higher than those of previous studies in Korea which did not incorporate the above tests, was similar to that of other countries. DEB and MMC tests were readily feasible and useful in screening FA in patients with AA as well as other bone marrow failure syndromes. A nation-wide screening and registry for FA should be initiated since FA requires different therapeutic and management options from idiopathic AA.

Key Words: *Fanconi's anemia; Anemia, aplastic; Cross-linking agents; Myelodysplastic syndromes; Mass Screening; Korea; Child*

Hoon Kook, Deok Cho,* Sung Ho Cho, Won Pyo Hong,* Chan Jong Kim, Ji Young Park, Won Sang Yoon, Dong Wook Ryang,* Tai Ju Hwang

Departments of Pediatrics and Clinical Pathology*, Chonnam National University Medical School, Research Institute of Medical Sciences, Kwangju, Korea

Received: June 5, 1998
Accepted: July 22, 1998

Address for correspondence
Hoon Kook, M.D.
Department of Pediatrics, Chonnam National University Medical School, 8 Hak-dong, Dong-gu, Kwangju 501-757, Korea
Tel: +82.62-220-6645, Fax: +82.62-222-6103
E-mail: hoonkook@chonnam.chonnam.ac.kr

*This work was supported by the Research Grant from Chonnam National University Hospital Clinical Research Institute (95-Gen-2).

INTRODUCTION

Aplastic anemia (AA) is a heterogeneous group of disorders characterized by loss of hematopoietic cells, fatty replacement of the marrow and pancytopenia. Fanconi's constitutional aplastic anemia (FA), the most common form of inherited bone marrow failure syndrome, is an autosomal recessive disorder characterized by the development of pancytopenia by the mean age of 6-8 years, and characteristic congenital anomalies (1). However, as about 30% of FA patients may be relatively normal in appearance, the correct diagnosis of FA is no longer relied upon physical anomalies or the development of AA (2). Currently in Western countries, patients are identified by characteristic breaks in the chromosomes of peripheral blood lymphocytes following clastogenic stress, such as diepoxybutane (DEB) or mitomicin C (MMC) (2-4).

The incidence of FA has been reported to constitute 25% to 30% of pediatric aplastic patients (1, 5). In Korea, however, the frequency of FA among patients with AA has not been systematically evaluated and has probably been underestimated as DEB and MMC tests have not been routinely incorporated in the evaluation of patients with AA (6, 7). We report here the results of DEB and MMC tests as screening for FA in patients with various bone marrow failure syndromes from southwestern Korea.

MATERIALS AND METHODS

The study population were patients with bone marrow failure syndromes who were diagnosed and followed up at the Department of Pediatrics, Chonnam National University Hospital. A total of 43 cases were evaluated (Table 1): AA, 24 (6 of them had physical anomalies pertinent to FA); Diamond-Blackfan anemia (DBA), 4 (2 of them were identical twins with congenital heart defects; one twin had polydactyly in addition); myelodysplastic syndrome (MDS), 6 (one of them had typical physical characteristics of FA); acute myelogenous leukemia (AML) with congenital anomalies, 2; siblings of FA patients diagnosed by DEB and MMC tests, 6. Among them, 28 were males and 15 were females. The median age was 72 months with the range of 0 to 192 months.

Table 1. Clinical characteristics of the patients

Patient	Sex	Age (mo)	Clinical impression	Associated anomaly
1	M	7	FA	Hyperpigmentation, café-au-lait spot, micrognathia, broad nasal bases, microcephaly, small eyes
2	M	72	FA	Café-au-lait spot, family history (+)
3	F	53	FA	Congenital dislocation of hip, polydactyly, café-au-lait spot
4	M	156	FA	Hyperpigmentation, café-au-lait spot, microcephaly, micrognathia, broad nasal bases, polydactyly, small eyes, developmental delay, mental retardation
5	M	47	FA	Café-au-lait spot, horseshoe kidney
6	M	2	FA/MDS	Hypopigmented macule, café-au-lait spot, hand & foot anomaly, flat nasal bridge
7	M	81	AA	Café-au-lait spot
8	M	71	AA	(-)
9	M	46	AA	(-)
10	F	134	AA	(-)
11	M	158	AA	(-)
12	F	59	AA	(-)
13	M	122	AA	(-)
14	F	146	AA	(-)
15	M	153	AA	(-)
16	M	81	AA	(-)
17	M	166	AA	(-)
18	M	119	AA	(-)
19	M	108	AA	(-)
20	F	101	AA	(-)
21	F	64	AA	(-)
22	M	148	AA	(-)
23	M	55	AA	(-)
24	F	101	AA	(-)
25	F	47	AA	(-)
26	F	3	DBA	Mitral valve prolapse, mitral insufficiency
27	F	2	DBA	Polydactyly, atrial septal defect
28	M	1	DBA	(-)
29	M	4	DBA	(-)
30	M	42	MDS	(-)
31	F	74	MDS	(-)
32	M	144	MDS	(-)
33	M	108	MDS	(-)
34	M	2	MDS	(-)
35	M	28	MDS	(-)
36	M	108	AML	Hyperpigmentation, microcephaly, micrognathia, mental retardation, small eye
37	M	19	AML	Down syndrome, complete AV septal defect
38	M	46	Sib. of 1	(-)
39	F	32	Sib. of 3	(-)
40	F	192	Sib. of 4	(-)
41	F	38	Sib. of 6	(-)
42	F	0	Sib. of 6	?
43	F	162	Sib. of 7	(-)

M, male; F, female; AA, aplastic anemia; DBA, Diamond-Blackfan Anemia; MDS, myelodysplastic syndrome; Sib, sibling.

DEB and MMC tests were carried out according to the methods described elsewhere (8-10) with minor modifications. Briefly, heparinized peripheral blood lymphocytes were stimulated with phytohemagglutinin (Gibco, USA) for 3- or 4-day cultures according to the standard procedure in a culture medium containing RPMI 1640 (Gibco, USA) supplemented with 15% fetal bovine serum and 1% penicillin-streptomycin. DEB (Aldrich, USA) was added at the final concentration of 0.1 μg/mL to the culture 48 hours before harvest. MMC (Sigma, USA) was added at the final concentration of 0.1 μg/mL to the culture 24 hours before harvest. Control cultures without DEB or MMC were prepared at the same time.

For prenatal diagnosis DEB and MMC tests were car-

ried out on chorionic villi obtained at the 10th gestational week (No. 42). The method was modified from Simoni's description (11, 12). Chorionic villi were separated from maternal tissue under sterile conditions and primary cultures were plated in Chang Medium (Hana Media, Inc.) at 37℃, in an atmosphere of 5% CO_2. Seventy-two hours after subculture, the growth medium was replaced with fresh medium containing DEB and MMC at 0.1 μg/mL, respectively.

Harvesting and slide preparations were performed according to standard methods for preparation of chromosomes using hypotonic solution, 0.075 M KCl, and methanol-glacial acetic acid as a fixative. Giemsa staining was applied.

Analyses were performed on 20 metaphase cells from untreated, DEB-treated and MMC-treated preparations, respectively. Chromatid and chromosome aberrations were scored according to the recommendations of the International System for Human Cytogenetic Nomenclature (13). Breaks, fragments, and deletions were scored as one break, and quadriradial figures as two events (8).

RESULTS

According to DEB and MMC sensitivity, there was a clear discrimination between the two groups; 7 patients showed hypersensitivity to both DEB and MMC, while the rest showed no hypersensitivity. Seven hypersensitive patients revealed increased chromosomal breaks/cell of 1.95±1.35 (mean±S.D.) (range 0.45 to 4.25) to DEB, and mean breakage/cell of 1.50±0.52 to MMC (range 0.90 to 2.50). The remaining 36 patients showed no increased hypersensitivity; chromosomal breaks/cell of 0.02±0.03 (range 0.00 to 0.15) to DEB, and 0.06±0.05 (range 0.00 to 0.15) to MMC. The values to both DEB and MMC were significantly different between the two groups ($P<0.001$), respectively, with no overlap between the two groups. The 7 hypersensitive patients were confirmed to have FA (Fig. 1). The results of chromosomal breakage studies of FA patients are summarized in Table 2. Increased spontaneous breakage was found in 4 among 7 FA patients with DEB-sensitivity (range 0.05 to 0.25), while spontaneous breakage was not found in 3 FA patients as well as non-FA patients. Thus, spontaneous breakage was unable to distinguish the two groups.

One patient with AA without apparent physical stigmata of FA except for several café-au-lait spots had increased chromosomal breakage to both DEB and MMC (No. 7). Moreover, two patients with DBA (patient No. 26 & 27) and two patients with AML (No. 36 & 37) who shared common congenital anomalies found in FA did not show hypersensitivity to either DEB or MMC. The siblings of proven FA patients (No. 38 to 43) were also evaluated as the recurrence rate for FA in other siblings

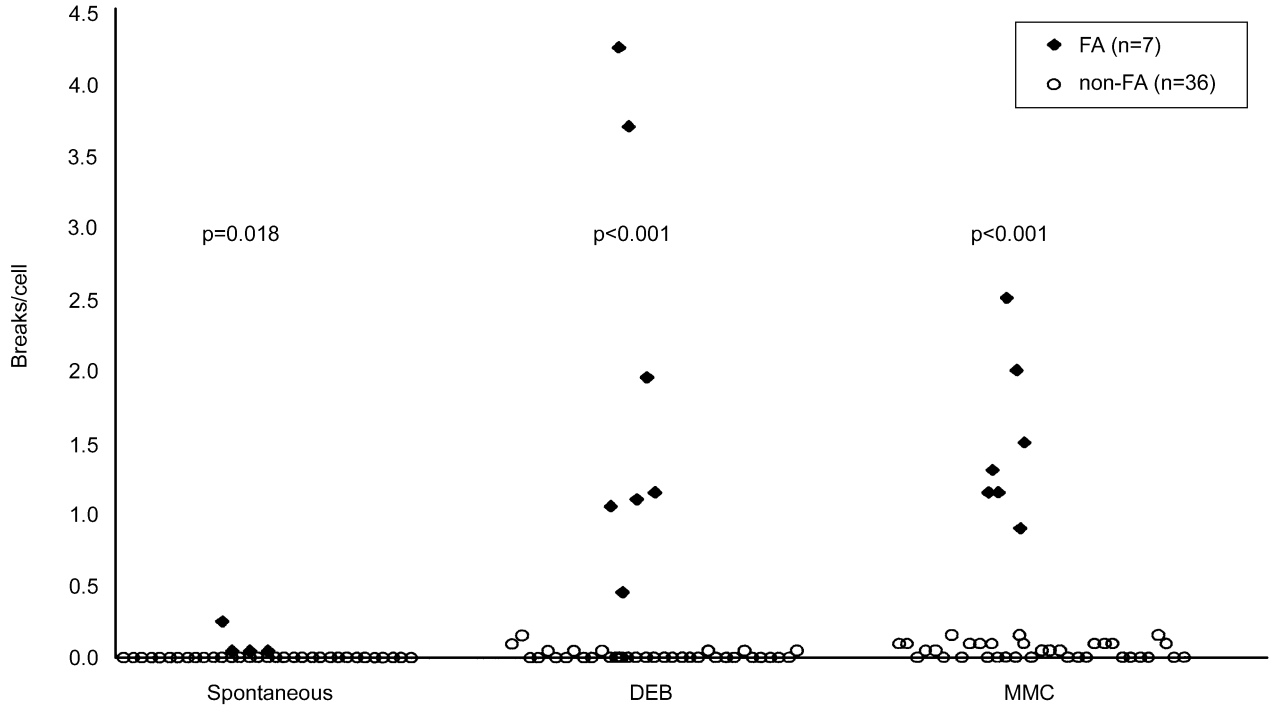

Fig. 1. Spontaneous and induced chromosomal breakage tests to diepoxybutane (DEB) and mitomicin C (MMC) in patients with Fanconi anemia (FA, ◆) and non-FA (○).

Table 2. Increased chromosomal breakage results in 7 patients with Fanconi anemia

Patient	Spontaneous breaks/cell	DEB breaks/cell	MMC breaks/cell
1	0.00	1.10	1.15
2	0.05	1.95	1.15
3	0.05	4.25	2.00
4	0.05	1.15	2.50
5	0.00	1.05	0.90
6	0.00	0.45	1.50
7	0.25	3.70	1.30
Mean	0.06	1.95	1.50
S.D.	0.08	1.35	0.52

is estimated to be 25% in autosomal recessive disorders. None of the siblings, including samples obtained by chorionic villi biopsy, was found to be a FA patient on the basis of the tests. Three FA patients (No. 1, 4, 7) were transplanted using HLA-identical siblings who were proven negative to DEB or MMC.

DISCUSSION

FA is a rare autosomal recessive disorder, with a high degree of clinical and genetic heterogeneity. Patients may exhibit congenital anomalies of any major organ system, or lack malformations and present with hematologic abnormalities such as bone marrow failure or AML (1, 2). However, diagnosis on the basis of clinical manifestations is often difficult and unreliable, due to the considerable overlap of the FA phenotype with that of a variety of genetic and nongenetic bone marrow failure syndromes. To support this we experienced a FA patient without typical physical anomalies except for café-au-lait spots (No. 7) and 2 other patients with DBA who shared common congenital anomalies of the two diseases (No. 26 & 27).

Diagnosis and differentiation of FA from other acquired AA or other similar inherited bone marrow failure syndromes, such as DBA are critical for many reasons: 1) About 30% of FA patients may be relatively normal in appearance. These patients were initially described as congenital AA type II or Estren-Dameshek AA (14), but later found to be variants of FA (8). 2) Treatment options are different as patients with FA do not respond to immunosuppressive treatments (8, 15), such as antithymocyte globulin or cyclosporin A, which have been commonly used and sometimes successful in patients with acquired AA (16, 17). On the other hand, patients with FA have a high rate of response to androgen therapy (2, 18). 3) Although the only available curative therapy for FA so far is a bone marrow transplantation (BMT) or umbilical cord blood transplantation (19-21), the conditioning regimen should be modified from that for acquired AA as the cells from FA patients are extraordinarily sensitive to alkylating agents and radiation (14, 22). 4) As FA is inherited in autosomal recessive fashion, each sibling donor of BMT should be screened for FA with the chance being 25%. 5) A curative measure should be attempted before the onset of leukemia which develops at a mean age of 14.5 years in about 10% of patients (23, 24). 6) The congenital anomalies in FA patients are not unique in FA patients, as DBA and thrombocytopenia absent radii syndrome may share some features of thumb and radial anomalies (25). Therefore, the diagnosis of FA is no longer relied upon the physical anomalies or the development of AA.

FA is a disorder that shows increased chromosomal fragility or cellular hypersensitivity to mutagenic chemicals, and developmental defects. Spontaneous chromosomal breakage can be seen not only in FA patients but in patients with Bloom's syndrome and ataxia telangiectasia. According to the International Fanconi's Anemia Registry (IFAR) report, however, the range of spontaneous chromosomal breakage in the FA group (0.02 to 1.9 breaks per cell with the mean of 0.27) was not significantly different from that found in normal controls or in the non-FA group (0 to 0.12 breaks per cell with the mean of 0.02). Thus baseline breakage frequency was proven to be not a useful method for discrimination of FA patients (8). In the present study, 3 FA patients did not show spontaneous chromosomal breakages.

Hypersensitivity of FA cells to the cytotoxic and clastogenic effects of DNA cross-linking agents provided a unique cellular marker for the disorder, which enabled it to distinguish itself from other chromosomal breakage syndromes. Numerous studies showed the sensitivity of FA cells to a variety of DNA cross-linking agents such as nitrogen mustard (26), cyclophosphamide metabolites (27), MMC (10) and DEB (8, 9).

Extensive experience with the DEB test has demonstrated high sensitivity, specificity and reproducibility of test results. According to the IFAR data, the mean$\pm$S.E. of breaks per cell for 104 FA patients was 8.96 ± 0.448, while that of 224 non-FA group was 0.06 ± 0.004 ($P<0.001$). There was no overlap in the range between the two groups. Similarly, the mean$\pm$S.E. (%) of cells with breaks in FA group was $85.15\%\pm1.99\%$, in contrast to $5.12\%\pm0.28\%$ in non-FA group ($P<0.001$) (8). In the current study, the chromosomal breakage of FA cells to DEB was distinguishable from non-FA patients (1.95 vs 0.02 breaks per cell) ($P<0.001$). The reason of discrepancy of mean breaks per cell between IFAR and current data is uncertain but small numbers of our cases, possible

modification of the test methods, or genetic susceptibility among different FA complementation groups might be responsible.

MMC has been used by other researchers in a clastogenic test for FA (10, 28). However, comparative studies have led to the choice of DEB as the agent most widely used for diagnosis of FA. Schroeder-Kurth et al. (29) compared DEB- and MMC-induced chromosomal breakage rates, and implied a significant potential for false-positive or false-negative diagnoses when MMC sensitivity was used as the diagnostic criterion because the overlap of the range of chromosomal breakage frequency between FA and non-FA individuals was found in at least 25% of cases. The high ratio of monoadduct production by MMC, a requirement for metabolic activation and instability in solution were all factors in the lack of reliability of MMC for FA diagnosis (3, 8). In this study, however, the result of MMC test was in accordance with that of DEB test without an overlap between FA and non-FA patients based on DEB test. The reliability of MMC test remains to be established in a larger cohort.

Although we analyzed 20 metaphases, some authors cautiously recommend more metaphases to be analyzed because of somatic mosaicism for DEB sensitivity which was found in up to 25% of FA patients (30). DEB-induced chromosomal breakage using cultured trophoblastic cells obtained by chorionic villi biopsy has been established as a reliable method for prenatal detection of FA in the first trimester of pregnancy (11, 12, 31). In this study, we could reliably exclude FA by prenatal testing in a mother having a FA patient.

As data from the IFAR showed that 3% and 9% of FA patients presented with MDS and AML, respectively, as initial manifestations of FA (23, 32, 33), children with MDS or AML, especially with typical congenital anomalies related to FA or family history of AA should be screened for FA with DEB test. In current study, all patients with pediatric MDS during the study period and 2 AML patients with congenital anomalies were screened for FA. A two-month-old patient with MDS (No. 6) with physical anomalies was found to have FA by DEB test.

The incidence of FA in Korean patients with pediatric AA has not been adequately evaluated (6, 7). However, the update of the observation from our institution signified that 8 were proven to be FA among 40 pediatric aplastic patients during the last 6.5 years (20%) (7). This estimation could not be the true frequency of FA among Koreans as there might be a chance of selection bias as our institution is a referral hospital and not all of the above 40 patients underwent DEB screening. This study calls for an urgent nation-wide screening and registry as FA poses unique challenges not only to patients and families, but to both clinicians and basic scientists.

REFERENCES

1. Alter BP. *Inherited bone marrow failure syndromes. In: Handin RI, Stossel TP, Lux SE, eds. Blood: Principles and Practice of hematology. Philadelphia: JB Lippincott, 1995: 227-91.*
2. Young NS, Alter BP. *Clinical features of Fanconi's anemia. In: Aplastic anemia acquired and inherited. Philadelphia: WB Saunders, 1994: 275-309.*
3. Auerbach AD. *Fanconi anemia diagnosis and the diepoxybutane (DEB) test. Exp Hematol 1993; 21: 731-3.*
4. Giampietro RF, Adler-Brecher B, Verlander PC, Pavlakis SG, Davis JG, Auerbach AD. *The need for more accurate and timely diagnosis in Fanconi anemia: a report from the International Fanconi Anemia Registry. Pediatrics 1993; 91: 1116-20.*
5. Windass B, Vowels MR, Hughes OD, White L. *Aplastic anaemia in childhood: prognosis and approach to therapy. Med J Aust 1987; 146: 15-9.*
6. Lee SI, Koh YY, Suh JK, Ahn HS, Hong CY. *Fanconi anemia. J Korean Pediatr Ass 1981; 24: 153-63.*
7. Cho SH, Kook H, Kim GM, Yoon WS, Cho TH, Hwang TJ. *A clinical study of Fanconi's anemia. Korean J Pediatr Hematol Oncol 1997; 4: 70-7.*
8. Auerbach AD, Rogatko A, Schroeder-Kurth TM. *International Fanconi Anemia Registry: relation of clinical symptoms to diepoxybutane sensitivity. Blood 1989; 73: 391-6.*
9. Auerbach AD, Adler B, Chaganti RSK. *Prenatal and postnatal diagnosis and carrier detection of Fanconi anemia by cytogenetic method. Pediatrics 1981; 67: 128-35.*
10. Cervenka J, Arthur D, Yasis C. *Mitomycin C test for diagnostic differentiation of idiopathic aplastic anemia and Fanconi anemia. Pediatrics 1981; 67: 119-27.*
11. Simoni G, Brambati B, Danesino C, Rossella F, Terzoli GL, Ferrari M, Fraccaro M. *Efficient direct chromosome analyses and enzyme determination from chorionic villi samples in the first trimester of pregnancy. Hum Genet 1983; 63: 349-57.*
12. Auerbach AD, Min Z, Ghosh R, Pergument E, Verlinsky Y, Nicolas H, Boue J. *Clastogen-induced chromosomal breakage as a marker for first trimester prenatal diagnosis of Fanconi anemia. Hum Genet 1986; 73: 86-8.*
13. Mitelman F. *ISCN: An International System for Human Cytogenetic Nomenclature. Basel: Karger. 1995.*
14. Estren S, Dameshek W. *Familial hypoplastic anemia of childhood: report of eight cases with in two families with beneficial effect of splenectomy in one case. Am J Dis Child 1947; 73: 671-87.*
15. Speck B, Gratwohl A, Nissen G, Leibundgut U, Ruggero D, Oseterwalder B, Burri HP, Cornu P, Jeannet M. *Treatment of severe aplastic anemia with antilymphocyte globulin or bone-marrow transplantation. Br Med J 1981; 282: 860-3.*
16. Rosenfeld SJ, Kimball J, Vining D, Young NS. *Intensive immunosuppression with antithymocyte globulin and cyclosporin as treatment for severe aplastic anemia. Blood 1995; 85: 3058-65.*
17. Matloub YH, Bostrom B, Golembe B, Priest J, Ramsay NKC.

Antithymocyte globulin, cyclosporin, and prednisone for the treatment of severe aplastic anemia in children. Am J Pediatr Hematol Oncol 1994; 16: 104-6.

18. Najean Y. *Androgen therapy in aplastic anaemia in childhood. In: Congenital disorders of erythropoiesis. CIBA Foundation Symposium 37. Amsterdam: Elsevier, 1976: 354.*
19. Gluckman E, Devergie A, Schaison G, Bussel A, Berger R, Sohier J, Bernard J. *Bone marrow transplantation in Fanconi Anaemia. Br J Haematol 1980; 45: 557-64.*
20. Guardiola P, Socie G, Pasquini R, Dokal I, Ortega JJ, van Weel-Sipman M, Marsh J, Locatelli F, Souillet G, Cahn JY, Ljungman P, Miniero R, Shaw J, Vermylen C, Archimbaud E, Bekassy AN, Krivan G, Di Bartolomeo P, Bacigalupo A, Gluckman E. *Allogeneic stem cell transplantation for Fanconi anaemia. Bone Marrow Transplant 1998; 21: S24-7.*
21. Gluckman E, Broxmeyer HE, Auerbach AD, Friedman HS, Douglas GW, Devergie A, Esperou H, Thierry D, Socie D, Lehn P, Cooper S, English D, Kurzberg J, Bard J, Boyse EA. *Hematopoietic reconstitution in a patient with Fanconi's anemia by means of umbilical cord blood from an HLA-identical sibling. N Engl J Med 1989; 321: 1174-8.*
22. Gluckman E, Auerbach AD, Horowitz MM, Sobocinski KA, Ash RC, Bortin MM, Butturini A, Camitta BM, Champlin RE, Friedrich W, Good RA, Gordon-Smith EC, Harris RE, Klein JP, Ortega JJ, Pasquini R, Ramsay NKC, Speck B, Vowels MR, Zhang M-J, Gale RP. *Bone marrow transplantation for Fanconi anemia. Blood 1995; 86: 2856-62.*
23. Butturini A, Gale RP, Verlander PC, Adler-Brecher B, Gillio AP, Auerbach AD. *Hematologic abnormalities in Fanconi anemia: an International Fanconi Anemia Registry study. Blood 1994; 84: 1650-5.*
24. Auerbach AD, Allen RG. *Leukemia and preleukemia in Fanconi anemia patients. A review of the literature and report of the International Fanconi Anemia Registry. Cancer Genet Cytogenet 1991; 51: 1-12.*
25. Hedberg VA, Lipton JM. *Thrombocytopenia and absent radii: a review of 100 cases. Am J Pediatr Hematol Oncol 1988; 10: 51-64.*
26. Berger R, Bernheim A, Le Coniat M, Veccione D, Devergie A, Gluckman E. *Nitrogen mustard-induced chromosome breakage: a tool for Fanconi's anemia diagnosis. Cancer Genet Cytogenet 1980; 2: 269-73.*
27. Berger R, Bernheim A, Gluckman E, Gisselbrecht C. *In vitro effect of cyclophosphamide metabolites on chromosomes of Fanconi anemia patients. Br J Haematol 1980; 45: 565-8.*
28. German J, Schonberg S, Caskie S, Warburton D, Falk C, Ray JH. *A test for Fanconi's anemia. Blood 1987; 69: 1637-41.*
29. Schroeder-Kurth TM, Zhu TH, Hong Y, Auerbach AD. *Variation in cellular sensitivities among Fanconi anemia patients, non-Fanconi anemia patients, their parents and siblings, and control probands. In: Schroeder-Kurth TM, Auerbach AD, Obe G, eds. Fanconi anemia: clinical, cytogenetic and experimental aspects. Heidelberg: Springer-Verlag, 1989: 105.*
30. Lo Ten Foe JR, Kwee ML, Rooimans MA, Oostra AB, Veerman AJ, van Weel M, Pauli RM, Shahidi NT, Dokal I, Roberts I, Altay C, Gluckman E, Gibson RA, Mathew CG, Arwert F, Joenje H. *Somatic mutation in Fanconi anemia: molecular basis and clinical significance. Eur J Hum Genet 1997; 5: 137-48.*
31. Auerbach AD. *Umbilical cord blood transplants for genetic disease: diagnostic and ethical issues in fetal studies. Blood Cells 1994; 20: 303-9.*
32. Auerbach AD, Weiner MA, Warburton D, Yeboa K, Lu L, Broxmeyer HE. *Acute myeloid leukemia as the first hematologic manifestation of Fanconi anemia. Am J Hematol 1982; 12: 289-300.*
33. Alter BP. *Leukemia in Fanconi anemia. Cancer Genet Cytogenet 1992; 58: 206-8.*