RESEARCH COMMUNICATION

Karyotyping in Retinoblastoma - A Statistical Approach

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Abstract

  Purpose: Karyotype analysis in hereditary retinoblastoma is considered to be of marginal value in risk prediction due to uncertainities in the assessment of 13q14 deletions. However, it is a low cost genetic test for retinoblastoma in developing countries. In the present study, the results of karyotype analysis were refined by a statistical method to overcome limitations. **Methods**: Karyotype analysis was performed by trypsin - Giemsa banding and naked eye karyotyping for 33 bilateral, 25 unilateral and one regressed retinoblastoma patients. The percentage of metaphases with 13q14 deletions in each case was plotted on a scatter diagram. Normalization of the data was achieved by log transformation and the results were statistically analyzed by one-sample't' test using SPSS version 9.0. **Results**: Seven samples had 13q14 deletion percentages above the cutoff value. One-sample't' test showed significance (p< 0.001). By this method, two unilateral and five bilateral patients had 13q14 deletions, constituting 11.8 % of cases. **Conclusion**: For accuracy, statistical analysis should be considered as an adjunct in karyotyping.

  Key Words: log transformation - one-sample't' test - outlier test - scatter diagram - 13q14 deletion

Introduction

  Retinoblastoma arises due to loss of both copies of the RB1 gene in developing retinal cells. Genetic testing is crucial for accurate risk prediction for retinoblastoma in close relatives of probands. Of the genetic tests available, karyotype analysis can be performed in basic genetic testing laboratory and is cheaper than molecular genetic tests (mutation screening of RB1 gene and molecular deletion analysis). In a developing country like India, cost factors are a major influence. Additional requirements like parental DNA samples for molecular deletion also make this more complicated.

  Karyotype analysis of peripheral blood has been reported to detect chromosomal abnormalities in 7.5 - 8.0% of bilateral and 1.0 - 4.9% of sporadic unilateral retinoblastoma patients (Ejima et al., 1988). The most frequent chromosomal abnormality in retinoblastoma is interstitial deletion of 13q14 band where the RB1 gene is located (Yunis and Ramsay, 1978). Patients with genomic 13q14 deletions could pass the susceptibility to retinoblastoma to 50% of their offspring. Amare et al., (2004) proved a higher frequency of q14 fragile site expression in retinoblastoma patients with constitutional 13q14 deletions, indicative of inherent genomic instability.

  Gandhewar et al., (2004) have argued that conventional cytogenetic analysis is of only limited value for retinoblastoma diagnosis. The variability in trypsin digestion and the relative positioning of the chromosomes (lying bent or straight) in the metaphase influences decisions regarding a 13q14 deletion. Lack of complete understanding of the processes during various stages of chromosome preparations acts as a constraint in refining karyotype analysis to improve the accuracy (Claussen et al., 2002). However, chromosomal region specific protein swelling is hypothesized to be responsible for Giemsa banding (Claussen et al., 2002). Fluorescent in situ hybridization on metaphase preparations also require extended chromosomes. The microscope cost, the limited number of samples that could be analyzed and requirement of expertise restrict routine application of FISH. FISH analysis with RB1 probes is considered as an adjunct to conventional cytogenetic studies in hematological malignancies when abnormalities of 13q14 are involved (Juneau et al., 1998).

  Statistics has been widely used in medical research to convert non-normally distributed data to a normal distribution before applying tests of significance. Transformation of non-normally distributed data to Gaussian distribution has been used in G-protein coupled research

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(Freeman and Spina, 2004). An exact permutation test based on Baumgarter-Weiss-Schindler statistic B is suggested for asymmetric or heavily tailed distributions of microarray data (Neuhauser and Senske, 2004). Psychiatric clinical studies proved that proper statistical methods in analysis of skewed data containing zeros would result in more meaningful results and conclusions (Delucchi and Bostrom, 2004). Transformations were applied to skewed data to ensure approximately normal distribution in a study to derive reference intervals for common biochemical tests in pediatric age group patients (Brinkworth et al., 2004). Statistical application in genetic studies of retinoblastoma established Knudson's 'two- hit' hypothesis, which explained the mechanism of tumourigenesis in hereditary and non–hereditary retinoblastoma (Knudson, AG (Jr.), 1971). Segregation analysis suggested that retinoblastoma could occur due to delayed mutation or host resistance (Bonaiti-Pellie and Briard-Guillemot, 1981). By segregation analysis, Harini et al., (2001) showed sporadic inheritance for majority of retinoblastoma patients. Fijal et al., (2002) analyzed the phenotypic effects of the p53 gene mutation in colorectal tumors by an algorithm created by statistical modeling. Betinsky et al., (2003) used a statistical approach to analyze results of a molecular neuro-genetic study (with partially biased sample selection) to retain important samples for analysis. In the present study, application of statistical methods in refining karyotype analysis in retinoblastoma is discussed.

Methodology

Patients with retinoblastoma enrolled were selected following enucleation surgery or referred by an ophthalmologist for genetic testing/counseling. Prior to karyotype analysis, an informed consent was obtained from the parents of the proband after detailed pedigree analysis and counseling. A follow-up counseling was done after the karyotype reports were ready. The study was done with prior ethical approval by the hospital board / ethics committee. Blood for karyotype analysis was collected from 59 probands during enucleating surgery or at a subsequent visit to the hospital.

Peripheral blood culture was done by 72 hours technique using Rosewell Parker Memorial Institute medium (RPMI 1640). Metaphase slides were prepared by standard methods followed by GTG banding (Harini et al., 2001).

The metaphases were first scanned under low power magnification to select well-spread and optimally stained metaphases with elongated chromosomes. Twenty-five good quality metaphases were karyotyped for each patient under 100X objective and additional metaphases, if necessary. Chromosome identification and nomenclature was done according to the International system for Human Cytogenetic Nomenclature (Mitelman, 1994).

Chromosomes were identified based on the length, arm ratio and banding. Initially all D group (chromosome 13 containing group) chromosomes were identified and then segregated into pairs based on the banding. Banding was given preference compared to size of chromosomes in segregating the chromosomes into pairs within each group. The chromosome 13 pair was examined in detail to assess the length of the bands and sub-bands and compared. The metaphase is included in karyotyping if the length of segments other than 13q14 region is normal and exactly equal in length. If there is distinct size difference in q14 region between the two homologous chromosomes, it was reported as 13q14 deletion.

A diagram of metaphase was drawn indicating each chromosome as in a line with the correct relative position, size and orientation in the metaphase. To ensure accuracy the results were verified by independent observation of the two 13 chromosomes by minimum of three research scientists in an anonymous manner. In equivocal cases photograph of the pair of 13 chromosomes were taken and compared. The result of karyotype analysis was reported as total chromosome number, followed by the chromosomal constitution.

Statistical Analysis

Considering the wide variation observed in staining of chromosomal segments and since majority of the chromosome preparations were in mid metaphase (condensed chromosomes), a statistical approach was taken to estimate 13q14 deletion in this study. In addition, in most of the patients (with 13q14 deletion) in this study, 13q14 deletion was apparent only in less than 50% of the metaphases.

The 13q14 deletion frequency (percentage of metaphases) in each case was entered into an excel worksheet for the 59 patients (Table 1). In the 19 cases without apparent 13q14 deletion, for statistical analysis the percentage of 13q14 deletion was taken as 0 %. Outlier test was used to find whether the 13q14 deletion (percentages) data followed normal distribution. Since the data was not normally distributed, log transformation was done to convert the 13q14 deletion frequency to normal distribution. The log transformation values were entered and the 13q14 deletion cutoff value at 10, 20, 25, 50, 75 and 90 percentiles were calculated by SPSS statistical package version 9.0. Nearly 10 % of retinoblastoma patients have cytogenetically visible 13q14 deletions (Bunin et al., 1989) and hence the 90th percentile of 13q14 deletion frequency (log transformation value) is taken as the cutoff for considering 13q14 deletion as true deletion (Table 1). A subject with log transformation value above cutoff was taken as 13q14 deletion and value below was not considered as deletion. The results were analyzed by one-sample 't' test for significance.

Results

Karyotype analysis by 72-hour peripheral blood culture and high resolution GTG banding was conducted for 59 retinoblastoma patients - 33 bilateral (55.9%), 25 (42.3%)

Table 1. Summary of Karyotype Analysis Results

S No.	Sample ID.	13q14 deletion(%)	Log transformation	Result
1	N:44	3.6	0.6628	-
2	L:66	6.5	0.8751	-
3	L:73	6.6	0.8808	-
4	V:81	11.5	1.0969	-
5	L:65	11.8	1.1072	-
6	R:9	13.8	1.1703	-
7	P:54	15.6	1.2201	-
8-10	W:49/Q:4/R:38	16.0	1.2304	-
11	N:68	16.7	1.2472	-
12	G1:26	18.5	1.2900	-
13	S1:78	18.5	1.2900	-
14-16	T:48/R:13/Z:61	20.0	1.3222	-
17-22	R:79/S:26/N:68			
	P:64/Q:13/S:45	24.0	1.3979	-
23	M:38	25.8	1.4281	-
24	N:70	26.0	1.4314	-
25	Q:33	27.0	1.4472	-
26	I1:4	27.9	1.4609	-
27-29	D1:69/C1:66/Q:5	28.0	1.4624	-
30	T:25	32.0	1.5185	-
31	X:35	33.3	1.5353	-
32	P:63	34.6	1.5514	-
33	Z1:71	34.8	1.5539	-
34	R:24	36.0	1.5682	+
35	N52	36.0	1.5682	+
36	Q:35	40.0	1.6128	+
37	L:39	40.9	1.6222	+
38	L:69	41.7	1.6304	+
39	N:78	56.0	1.7559	+
40	W:48	80.0	1.9085	+
Percentiles/ Cutoff values	50/1.2304	75/1.4054	90/1.5682	

NOTE: + indicate 13q14 deletion and – indicate no deletion. Log transformation values above 1.5682 indicate 13q14 deletion. Nineteen samples (not shown) had 13q14 deletion 0 %.

unilateral and one regressed (1.69%). The 13q14 deletion frequencies (in percentages) and the log transformation values for the 59 samples and the cutoff values derived by statistical analysis are shown in Table 1. The cutoff values at 5, 10 and 25th percentiles were zero. The cutoff at 90th percentile was derived as 1.5682 (Table 1). Outlier testing showed normal distribution of the log transformation values (range 0.0 - 1.9085) and mean (0.93 ± 0.6745). Seven samples had log transformation values above the cutoff (1.5682) (Table 1).

By this method, two unilateral (R24 and W48) and five bilateral patients (L39, L69, N52, N78 and Q35) had 13q14 deletion constituting 11.8 % of the retinoblastoma patients who underwent karyotype analysis. Analysis of the results by one - sample 't' test showed significance (p< 0.001).

Discussion

In retinoblastoma genetic screening, karyotype analysis is the simplest test providing results within few days of receipt of blood sample. Karyotype analysis is the simple

and affordable test to most of the retinoblastoma families. Other advantages of karyotype analysis are, easy to master, stable characteristics of the chromosomes with clearly defined abnormal states, possibility of preservation of slides for future applications (mounted slides or in fixative), detection of multiple chromosomal abnormalities from a single specimen, interpretation of results without simultaneous parental testing (required in molecular cytogenetic deletion analysis) and quality of culture could be assessed during incubation and subsequent processing of the blood specimen by naked eye examination.

Yunis and Ramsay, (1978) concluded that sub-bands of 13q14 region should be carefully analyzed for minute deletion in retinoblastoma patients. Cells with well-banded elongated chromosomes in mid-metaphase or early metaphase are necessary for evaluation of 13q14 deletion. In a well-banded elongated metaphase, the light bands q12, q14 and dark bands q21 and q31 measure 80 % of length of q arm of chromosome 13 and 13q14 deletion could be easily ascertained. Yunis and Ramsay, (1978) and Motegi, (1981) showed that, if the chromosomes are condensed in mid or early metaphase, determining whether 13q14 deletion is present, is difficult. The extent of trypsinisation which in-turn affects the differential Giemsa staining of the chromosomal segments could also influence the decision whether 13q14 deletion is present. Statistical analysis was done assuming that these confounding factors might have influenced the karyotype results equally for the 59 blood samples.

In the present study, in all cases, 13q14 deletion was apparent only in less than 50% of metaphases with frequency (0 – 80.0 %) and mean 17.47 (Table 1). Only in one unilateral patient, 13q14 deletion was apparent in 80% of metaphases. Delucchi and Bostrom, (2004) reviewed the statistical methods to analyze heavily skewed psychiatric clinical study data containing many zeros values. In this study, zero values and non-zero values were analyzed using separate statistical models and showed that use of proper statistical methods would result in more meaningful results and conclusions (Delucchi and Bostrom, 2004). In the present study in 19 samples, 13q14 deletion was not apparent in none of the metaphases karyotyped and hence 13q14 deletion frequency was taken as 0%. The 13q14 deletion frequency (percentage of metaphases with apparent 13q14 deletion) was calculated in each case and the percentile distribution was derived using SPSS version 9.0 after log transformation. Log transformation was done to convert the 13q14 deletion frequencies (that did not follow normal distribution) to log transformation values (followed normal distribution). Log transformation values showed a scatter diagram with normal distribution with Outlier test. The 90th percentile value i.e. 1.5682 was taken as the cutoff for 13q14 deletion and hence taken as the test value in the analysis by one-sample 't' test (Table 1). One-sample't' test showed significance (p < 0.001). In this way cytogenetically visible deletion of RB1 gene (13q14 deletion) by Giemsa banding was detected in two unilateral (R24 and W48) and five bilateral patients

(L39, L69, N52, N78 and Q35) constituting 11.8 % of patients.

Deletion involving 13q region are reported in 13q⁻ syndrome, hematological malignancies like multiple myeloma, Polycythemia vera, chronic lymphocytic leukemia, and breast cancer and osteosarcoma. The statistical analysis used in the present study could be useful in these disorders also. The interdisciplinary approach adopted might be useful in deriving maximum benefit out of the existing techniques for reporting.

Karyotyping is the simplest and affordable genetic test for most of the retinoblastoma families especially in a developing country like India. In the present study 33 bilateral and 25 unilateral and one regressed retinoblastoma patients were karyotyped by Giemsa banding. Cytogenetically visible deletion of RB1 gene (13q14 deletion) by Giemsa banding was detected in two unilateral and five bilateral retinoblastoma patients (11.8%). Karyotype analysis should be interpreted by taking into consideration the technical limitations like extent of elongation of the chromosomes, variation in trypsinisation and staining intensity of the chromosomal segments and the number of metaphases with 13q14 deletion. A statistical approach could be used as an adjunct in deriving true 13q14 deletion results after karyotype analysis. The statistical approach taken in the current study improvises karyotype analysis and might be useful in other diseases where chromosome 13 deletions are involved.

Acknowledgements

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