Polycythemia vera: analysis of DNA from blood granulocytes using comparative genomic hybridization

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Background and Objectives. The diagnosis of polycythemia vera (PV) is supported by the finding of an abnormal karyotype in patients with erythrocytosis. However, most PV patients have normal marrow cytogenetics at presentation and there is reluctance to use this test routinely. Comparative genomic hybridization (CGH) is a cytogenetic screening technique that analyzes interphase cells. This approach offers practical advantages over conventional cytogenetics and interphase fluorescence *in-situ* hybridization (IFISH). We have therefore evaluated the diagnostic utility of CGH applied to blood granulocytes in PV.

Design and Methods. Blood granulocytes from 17 PV patients were analyzed using CGH and the results compared with those from previous conventional cytogenetics and IFISH studies.

Results. Three patients had abnormal CGH profiles. One case had gain of 9p. This patient had normal IFISH results using a centromere-9 probe. The second case had complete gain of chromosomes 8 and 9 and the third had complete gain of chromosome 9, all confirmed by IFISH. Cytogenetics had not been performed in two of these cases and had failed in the third. Three cases with 20q deletion according to cytogenetics and/or IFISH, were normal by CGH. The remaining subjects were normal by all methods.

Interpretation and Conclusions. CGH analysis of blood granulocytes can detect the chromosome gains commonly observed in PV. However, CGH cannot be relied on to detect 20q deletions, which are the most frequent cytogenetic abnormality in PV. Thus, CGH has a role in the diagnosis and follow-up of PV patients, but must be used in conjunction with other methods.

Key words: polycythemia vera, comparative genomic hybridization

Polycythemia vera (PV) is a chronic clonal neoplastic multilineage myeloproliferative disorder with a predisposition to transform into acute leukemia.[1] The diagnosis of PV rests largely on the exclusion of apparent and secondary erythrocytoses.[2] Positive indicators of PV, such as endogenous erythroid colonies, low serum erythropoietin, neutrophilia, splenomegaly, and thrombocytosis, are not specific and are not present in all cases.[3-5] The presence of an abnormal karyotype, a strong positive marker of PV, is therefore a valuable finding in the differential diagnosis of erythrocytoses.[2] However, the majority of PV patients have normal bone marrow metaphase cytogenetics at presentation[6] and there is a reluctance to use these demanding procedures in routine practice.

We have previously demonstrated that specific karyotype abnormalities in PV patients can be revealed using interphase *in situ* hybridization (IFISH) analysis of blood granulocytes.[7] Such an approach has significant practical advantages over conventional cytogenetics as it obviates the need for bone marrow biopsy and does not require the eliciting of mitoses from the neoplastic clone. However, IFISH is limited by the availability of probes and the necessity to pre-select markers for analysis. Comparative genomic hybridization (CGH) is a molecular cytogenetic technique that, like IFISH, is applicable to interphase cells, but, unlike IFISH, provides an assessment of the entire genome.[8] We have, therefore, evaluated the diagnostic utility of CGH in PV, using blood granulocytes as a source of myeloid DNA.

Design and Methods

Patients

The study group comprised 17 PV patients (13 men and 4 women), aged 28 to 81 years (mean 63.3,

median 63.1 years), who had presented between 1 and 176 months (mean 85, median 106 months) prior to this study. The patients were a random selection from our clinics and all satisfied the MPD(UK) Study Group diagnostic criteria for PV.[2,4] Two healthy laboratory staff (one male, aged 40 years, and one female, aged 61 years) provided blood for the preparation of normal DNA.

Preparation of granulocyte DNA and comparative genomic hybridization

Granulocytes were isolated from peripheral blood by density centrifugation as previously described[7] and their DNA extracted by standard methods. Samples of DNA were subsequently labeled using SpectrumGreen™ (test DNA) or SpectrumRed™ (normal DNA) fluorescent 2'-deoxyuridine-5'-triphosphate by nick-translation according to the supplier's (Vysis) protocol. CGH probes were prepared by co-precipitating SpectrumGreen™-test DNA (400 ng), gender-matched SpectrumRed™-normal DNA (400 ng), COT-1® DNA (26.6 µg, Life Technologies) and salmon sperm DNA (10 µg, Sigma) using 0.1 and 2.5-fold volumes, respectively, of sodium acetate (3.0M) and ethanol at –70ºC followed by centrifugation at 4ºC. The DNA pellets were dried, dissolved in 10 µL of hybridization buffer (50% v/v formamide, Fluka, 10% w/v dextran sulfate, Sigma, in 4XSSC), denatured at 73ºC for 5 minutes and pre-annealed at 37ºC for 60 to 120 minutes. CGH target metaphase slides, prepared using acetic acid-methanol (1:3) fixed phytohemagglutinin-stimulated normal male lymphocytes, were pre-treated using pepsin and 2XSSC then denatured in formamide (70% v/v in 2XSSC) at 73ºC as previously described.[7] The CGH probe was applied to the CGH target slide, sealed under a coverslip and the slide incubated at 37ºC for 72 hours. Post-hybridization washes consisted of 0.3% v/v NP-40 (Merck) in 0.4XSSC at 74ºC for 2 minutes followed by 0.1% v/v NP-40 in 2XSSC at room temperature for 1 minute. The slides were then dried, mounted under a 4'-6'-diamidine-2-phenylindole dihydrochloride (DAPI)-containing medium (Vector Laboratories) and subsequently analyzed using a CytoVision™ workstation (Applied Imaging). Fluorescence and DAPI images were captured from 12 metaphases per slide for the generation of composite CGH profiles. Chromosome classification was validated by two authors. Deletions and amplifications were suspected when the slide average green:red fluorescence ratio for a given chromosomal region lay outside the range 0.8 to 1.2. Suspected abnormalities were confirmed by repeat CGH testing and, when appropriate, by IFISH analysis of acetic acid-methanol fixed granulocytes, as previously described.[7]

Results

The results are presented in Table 1. Three of the 17 patients tested (17.6%) had an abnormality by CGH. Case #7 was found to have a gain of material from the p arm of chromosome 9 [rev ish enh(9p)] (Figure 1). This patient had been previously found to have normal IFISH results using a chromosome-9 centromere probe. The second patient (case #13) was

Table 1. Results.

Case#	Age at time of study (yrs)	Months since presentation	Neutrophils ($\times 10^9$/L)	Platelets ($\times 10^9$/L)	Previous cyto-reductive therapy	Cyto-reductive therapy at the time of study	Previous conventional cytogenetics	Previous IFISH* (% abnormal nuclei)	CGH
1	81	119	6.4	325	V	V	NT	NAD	NAD
2	62	2	8.5	364	V	V	NAD	NT	NAD
3	66	34	3.0	270	HV	HV	20q deletion	20q deletion (63%)	NAD
4	81	164	12.0	422	HV	H	NT	NAD	NAD
5	63	126	11.0	283	BHV	H	NT	NAD	NAD
6	53	118	5.2	379	BH	H	NT	NT	NAD
7	57	176	13.8	613	BHV	H	NT	20q deletion (50%)	Gain of 9p
8	64	47	13.5	306	HV	H	NT	NAD	NAD
9	56	106	15.5	165	HV	HV	NAD	NAD	NAD
10	28	34	5.3	513	HV	H	NAD	NAD	NAD
11	73	5	7.6	235	V	V	20q deletion	20q deletion (72%)	NAD
12	61	114	6.5	527	BHV	H	NT	NAD	NAD
13	58	1	13.5	242	V	V	Failed	NT	Gain of 8 and 9
14	69	123	26.0	344	BHV	H	NT	Trisomy 9 (69%)	Gain of 9
15	62	92	11.0	294	HV	H	NT	NAD	NAD
16	66	19	8.2	504	HV	H	NAD	NAD	NAD
17	78	158	4.9	224	BH	Nil	NT	NT	NAD

*Therapy. B: busulphan, H: hydroxyurea, V: venesection. Results. NT: not tested. NAD: no abnormality detected. *IFISH involved probes for centromere 8, centromere 9, to detect trisomies 8 and 9, and a probe for locus D20S108, to detect 20q deletions. These results have been reported previously.[7]*

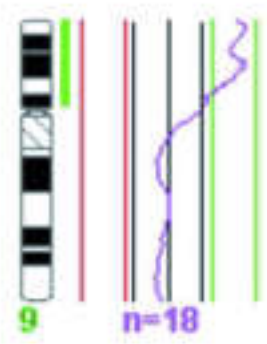

Figure 1. Case 7: CGH profile of chromosome 9. The magenta line represents the mean green:red fluorescence ratio of 18 captured chromosomes 9 hybridized with a CGH probe containing SpectrumGreen™ labeled DNA from case 7. The three vertical straight black lines represent (from left to right) the 0.8, 0.0 and 1.2 green:red fluorescence ratios.

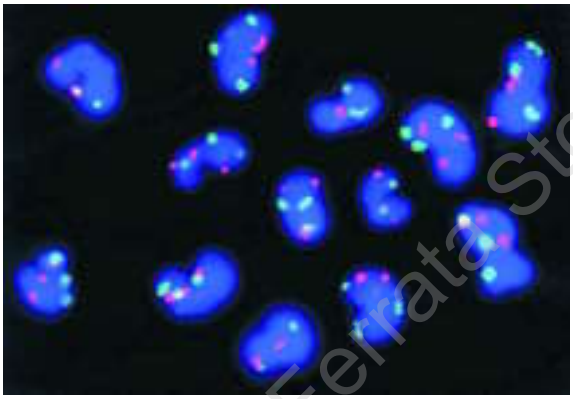

Figure 2. Case 13: IFISH assay for centromeres 8 and 9. α-satellite probes for centromeres 8 (red fluorescence) and 9 (green fluorescence) were hybridized to fixed granulocytes from case 13.

found to have gains of material from the whole of chromosome 8 and the whole of chromosome 9 by CGH [rev ish enh(8,9)]. An IFISH assay using probes for centromeres 8 and 9, which had not been previously performed in case #13, revealed that 66% of this patient's granulocytes exhibited both trisomy 8 and trisomy 9 [nuc ish 8cen(D8Z2×3), 9cen(D9Z1×3)] (Figure 2). Conventional cytogenetics on case #13, attempted on a bone marrow aspirate taken at the same time as samples were obtained for CGH and IFISH, failed due to lack of metaphases. The CGH profile of the third patient (case #14) showed a gain of material from the whole of chromosome 9 [rev ish enh(9)], consistent with an earlier IFISH assay using centromere probes which had demonstrated trisomy 9 [nuc ish 9cen(D9Z1×3)] in 69% of blood granulocytes. Three patients had been previously identified as having a deletion of 20q on the basis of hemizygous D20S108 deletion by IFISH (cases #3, 7 and 11) and by conventional cytogenetics (cases #3 and 11). The CGH assay however, failed to identify any abnormality of chromosome 20 in these three patients. The earlier IFISH studies had indicated that 50% to 72% of circulating granulocytes carried the D20S108 deletion in these patients (Table 1).

When the previous IFISH and present CGH results were considered together, 5 of the 17 patients studied (29%) had one or more abnormality. This number of cases is clearly too small to allow definitive correlations with clinical or laboratory parameters to be made. However, as shown in Table 2, it is apparent that the group with abnormalities was broadly similar to those without in respect of the noted parameters. All patients were clinically stable and none exhibited signs of transformation to myelofibrosis or acute leukemia at the time of study.

Table 2. Characteristics of patients with and without IFISH and / or CGH abnormalities.

	*Abnormal**	*No abnormality detected*
Number of cases	5	12
Age, years (mean, median, range)	64, 66, 57-73	63, 63, 28-81
Months since presentation (mean, median, range)	68, 34, 1-176	92, 110, 2-164
Number of cases with neutrophilia (>10×10⁹/L)	3 (60%)	5 (42%)
Neutrophil count, ×10⁹/L (mean, median, range)	12.8, 13.5, 3.0-26.0	9.0, 8.4, 4.9-15.5
Number of cases with thrombocytosis (>400×10⁹/L)	1 (20%)	3 (25%)
Platelet count, ×10⁹/L (mean, median, range)	341, 270, 235-613	359, 345, 165-527
Number of patients receiving chemotherapy at time of study	3 (60%)	9 (75%)
Number of patients who had received chemotherapy prior to this study	3 (60%)	10 (83%)

**The combined results of previous IFISH and present CGH studies.*

Discussion

The diagnosis of PV is strongly supported by the demonstration of clonal myelopoiesis.[4] Two approaches have been used for this purpose, namely analysis of X chromosome inactivation patterns (XCIPs)[9] and conventional cytogenetics.[6] However, neither approach is satisfactory. Clonality assays based upon XCIPs, which are only applicable to females, must be interpreted with caution because apparently clonal results can be observed in some normal elderly women.[10,11] Acquired karyotypic abnormalities are more reliable markers of clonality than XCIPs but are present in only about one third of PV patients and the frequency is even lower at diagnosis.[6] Thus, the differential diagnosis of erythrocytoses and the monitoring of PV patients would be greatly enhanced if sensitive, robust and more widely applicable assays of clonality were available.

CGH is a molecular cytogenetic method that screens the entire genome of interphase cells for amplifications and deletions of DNA sequences.[8] In brief, test and normal genomic DNAs are isolated, labeled separately using different fluorochromes, mixed in equal amounts, denatured and applied to a slide containing denatured normal metaphase chromosomes. During incubation the test and normal DNAs compete with each other for complimentary sequences in the normal metaphase chromosomes. When there is amplification of DNA sequences in the test sample, for example due to a trisomy, more test DNA binds to the corresponding region of the chromosome than normal DNA. Conversely, when there is DNA deletion in the test, more of the normal DNA hybridizes to the target chromosome. Thus, CGH is a dosage assay and it cannot detect balanced chromosomal rearrangements. The relative amount of test:normal DNA binding is determined by analysis of digital images captured during fluorescence microscopy. Several metaphases are captured and an average test:normal DNA fluorescence ratio derived for each chromosome in order to exclude random variations in hybridization. Thus, CGH is potentially an ideal clonality screening tool as it does not require pre-selection of loci for analysis, is applicable to men as well as women, and does not require the production of metaphases from the tumor. We, therefore, evaluated the diagnostic utility of CGH as a clonality assay in PV. A particular attraction of CGH was the facility to study circulating granulocytes, which we have previously shown to be suitable for the demonstration of specific cytogenetic abnormalities in PV by IFISH,[7] thereby overcoming the necessity to obtain bone marrow samples.

Of the 17 patients we studied, 3 (17.6%) were found to have an abnormality by CGH.

The first case had a gain of material from the p arm of chromosome 9. Partial gains of chromosome 9 have been previously reported in PV in the forms of isochromosome $9p^{12}$ and in unbalanced translocations between 9p and chromosomes $1^{12\text{-}16}$, 2^{17} and 18^{12} or between 9q and chromosome 1.[16,18] In our patient, the gain of 9p material was the sole abnormality in the CGH profile and therefore the exact cytogenetic nature of the aberration remains undefined. This patient had been previously tested by IFISH using a probe for centromere 9 and found to be normal. Clearly, partial amplifications and deletions that do not involve centromeric regions will not be detected using α-satellite chromosome enumeration probes. This case therefore illustrates the potential superiority of CGH over IFISH as a screening tool because CGH provides an assessment of the entire genome in one assay, without the need to pre-select regions for scrutiny.

The second case with an abnormal CGH profile had complete gain of chromosomes 8 and 9. Subsequent IFISH analysis confirmed the presence of a population with simultaneous trisomy 8 and trisomy 9, a well-documented abnormality in PV.[19,20] Conventional bone marrow cytogenetics, attempted on a sample taken at the same time as the CGH/IFSH sample was obtained, failed due to poor sample quality. This case therefore illustrates the practical utility of CGH, which can be applied to blood cells and archived DNA samples and is thus a useful back-up tool when conventional methods fail.

Our third patient with an abnormality by CGH had gain of chromosome 9, confirming an earlier IFISH result, which had demonstrated complete trisomy 9.

In contrast to the successful identification of chromosomal gains in our patients, CGH failed to detect deletion of 20q that had been previously identified in three patients by locus-specific IFISH testing of blood granulocytes (3/3 cases) and by marrow cytogenetics (2/2 cases). This outcome is consistent with the relatively poor sensitivity of CGH, which is acknowledged to be a major deficiency of the technique.[8] The lower limit for detection of DNA deletions by CGH is about 10Mb, provided the sample has a high percentage of abnormal cells.[21] Cytogenetically visible 20q deletions in myeloproliferative disorders involve either one or both Giemsa-positive bands on this small F-group chromosome.[22] At a molecular level, the smallest reported 20q deletion is 5 to 6 Mb[23] but the majority of PV patients have 20q deletions of >10Mb.[24] Thus, it is possible, but unlikely, that our three cases had 20q deletions below the 10Mb sensitivity limit of CGH. A more plausible explanation for our failure to detect the 20q deletion by CGH might be have been the presence of normal granulocytes in

these samples. 20q deletion is the single most common cytogenetic abnormality in PV[6] and the possibility that this deletion will not be detected by CGH must, therefore, be viewed as a serious drawback. Improved sensitivity of CGH may be possible by replacing normal metaphases by arrays of cloned DNA as the hybridization target.[8] An evaluation of such an approach for use in PV is warranted. Fortunately, the interstitial deletions of 20q associated with PV are readily detectable by microsatellite PCR[25] and by IFISH,[7] and these methods should therefore be employed alongside CGH when investigating an individual patient.

In summary, our findings, albeit on a relatively small sample of patients, provide a case for using CGH in the diagnosis of PV. The ability to detect partial chromosomal gains, to identify various abnormalities in one assay without the need for extensive probe panels and the applicability of the technique to peripheral blood cells strongly commend this technique as a screening tool. However, the inability of this method to detect balanced rearrangements, the requirement for a high frequency of abnormal cells and the relatively poor detection limit of 10Mb mean that CGH alone cannot be relied on to provide a comprehensive cytogenetic profile. Thus, CGH should be employed as an adjunct to conventional cytogenetics and could be particularly helpful when marrow samples are not available or are of inadequate quality.

Contributions and Acknowledgments

NBW: study conception and design, CGH and IFISH analysis and interpretation of data, manuscript preparation, revision and final approval. AMGW and SA: CGH analysis and interpretation of data, manuscript revision and final approval. TCP: study conception and design, manuscript revision and final approval. The authors are listed in descending order of their contribution to the work.

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Disclosures

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Manuscript processing

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Potential implications for clinical practice

The present study demonstrates that comparative genomic hybridization applied to DNA from circulating granulocytes is a practical and efficacious method for detecting certain karyotype abnormalities in patients with polycythemia vera. However, this molecular cytogenetic method may not be sufficiently sensitive to detect small changes, such as 20q deletions, and consequently must not be used alone in the diagnostic cytogenetic evaluation of individual patients.

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