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Immunoglobulin heavy and light chains and T-cell receptor beta and gamma chains PCR assessment on cytological samples. A study comparing FTA cards and cryopreserved lymph node fine-needle cytology

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Objectives: To evaluate and compare the DNA yield and quality extracted from lymph node fine needle cytology (FNC) samples stored on FTA cards to those cryopreserved, and to assess the immunoglobulin heavy and light chains (IGHK) and T-Cell receptor beta and gamma chains (TCRBG) PCR tests.
Methods: DNA extractions were performed on FNC of 80 non-Hodgkin lymphomas (NHL), four myelomas and 56 benign reactive hyperplasias (BRH) cryopreserved and stored on FTA cards. The JAK2 gene was amplified to assess the DNA integrity and the IGHK/TCRBG clonality status was tested.
Results: IGHK monoclonality was found in 99% of B-cell NHL and 100% of myeloma. TCRBG monoclonality was found in 100% of T-cell NHL. TCRBG polyclonality was detected in 97% of B-cell NHL, 100% of myeloma and 96% of BRH. IGHK/TCRBG PCR data were confirmed by histological and/or follow-up controls. No differences were found in the DNA quality between cryopreservation and FTA cards storage methods.
Conclusions: IGHK/TCRBG PCR of the lymphoproliferative process on FTA cards is comparable to those cryopreserved. FTA cards can be used to store lymph node FNC for further molecular investigations.

Keywords: IGH, IGK, TCRB, TCRG, clonality testing, non-Hodgkin lymphoma, fine needle cytology, FTA cards

Clonality testing

Clonality testing is an important step in the cytological diagnosis of non-Hodgkin lymphoma (NHL). In fact, in the absence of histological criteria and/or of significant cytological atypia, clonality testing may be the only diagnostic criteria on cytological samples. Clonality can be determined by identifying different and specific chromosomal abnormalities, such as the t(14,18)(q32;q21) in follicular lymphoma (FL) or the t(11;18)(q21;q21) in Mucosa associated lymphoid tissue (MALT) lymphoma using fluorescence *in situ* hybridization (FISH). Nonetheless, chromosomal abnormalities are usually investigated when a specific pathological entity is highly suspected or has already been diagnosed. Moreover, a small number of translocations is shared among different entities; therefore, specific chromosomal abnormalities cannot be conveniently used to determine clonality in different B-cell lymphoproliferative processes.[1] Clonality can also be assessed via the phenotypic quantification of light chain

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expression, mainly evaluated by flow cytometry (FC) and by specific phenotypic profiles, such as CD5/CD19, a high percentage of CD10/CD19 co-expression or loss of one of T-cell surface antigens CD2/CD3/CD7. Many NHLs lack these phenotypic profiles and a variable percentage of cases do not show light chain restriction or show lack of any expression at all.[2–4] Moreover, FC requires cell suspensions that are not always available; in these cases, clonality testing is an indispensable procedure.

Immunoglobulin/T-cell receptor (IG/TCR)

Immunoglobulin/T-cell receptor (IG/TCR) polymerase chain reaction (PCR) assessment is the main procedure, as well as the gold standard to determine clonality. Clonality testing is usually performed using DNA-PCR tests that are widely used for NHL, as the corresponding neoplastic cells have IG or TCR genes clonally rearranged. This feature has led to the development of different molecular tests that investigate the rearrangement status of these genes, using different primers and gene targets.[5–20] EuroClonality/BIOMED-2 is a multiple institutions consortium that has tested and validated primers and PCR systems for IG/TCR clonality testing and has produced guidelines for interpretation and reporting of results in suspected lymphoproliferative processes.[6] PCR has been validated and standardised for fresh or cryopreserved cell material, whereas formalin-fixed-paraffin-embedded (FFPE) tissues have also been proven and accepted as a reliable option.[15] Therefore, IG/TCR-PCR clonality can be determined on genetic material obtained from different samples, such as smears or fluids, even if the optimal samples are fresh or cryopreserved cells.[4–22] Fine needle cytology (FNC) and other cytological procedures are used in the diagnosis of lymphoproliferative processes and sometimes represent the only available diagnostic tool. FNC samples are generally scanty when compared with those obtained by thick needle or surgical biopsies, and the preservation and storage of genetic material affect clinical effectiveness.

FTA cards

Whatman Classic and Elute FTA cards (Whatman GE, Maidstone, UK) are filter papers that are impregnated with chemicals that lyse cell membranes and denature proteins. As a consequence, residual nucleic acids are framed, immobilised and stabilised in the cards and can be stored at room temperature in shielded re-sealable pouches. In this way, FTA cards protect nucleic acids from nucleases, oxidation, UV damage, microbial and fungal attacks, and can be used several times.[23–28] FTA cards have been used to collect and store genetic material obtained by scraping or FNC of surgical samples, or by *in vivo* FNC of lymph nodes.[23,24,26,27] The aim of this study was to evaluate whether DNA quality and yield, immunoglobulin heavy and light chains and/or T-Cell Receptor beta and gamma chains PCR clonality assessment (IGHK/TCRBG PCR) of lymphoproliferative processes, obtained by FNC and stored on FTA cards, are comparable with those obtained on cryopreserved cells. FNC/FC procedures and the IGHK/TCRBG PCR assessments were also compared to the histological/follow-up controls to evaluate the diagnostic efficacy of the two procedures.

Materials and methods

Fine needle aspiration, flow cytometry and DNA storing

One hundred and forty consecutive lymph node FNC were used in the present study. Corresponding patients were enrolled at the Azienda Ospedaliera Universitaria of the University of Salerno, from 1 January 2013 to 30 May 2015. Patients were previously informed about the procedure and signed an informed consent form, which included the use of diagnostic material for research purposes. The present study design had been previously approved by the Campania Sud Ethic Committee (cometicocampaniasud@asl3sud.it). Patients underwent ultrasound (US)-guided FNC that was performed as previously reported.[29] For each case, a cell suspension was prepared *in vivo* along with other diagnostic procedures.[30] The first smear was Diff-Quik stained and used for rapid on-site evaluation (ROSE); the others were fixed in 95% alcohol and used for Papanicolaou staining for immunocytochemical (ICC) analysis. A cell suspension was obtained by an additional pass; the FNCs were immediately flushed in 1000 μl of phosphate-buffered saline (Lonza Walkersville, Inc., Walkersville, MD, USA) and the needle was repeatedly rinsed by aspirating and flushing the fluid. Next, 600 μl of cell suspension was further diluted with PBS and used for flow cytometry immunophenotyping. The remaining concentrated 400 μl was vortexed and divided into two tubes:

240 μl was cryopreserved at −80 °C and the remaining 160 μl was spotted on the FTA card (Whatman™ Classic FTA™ Cards; GE Healthcare Ltd, Amersham Pl, UK, cat. n. WB120205). In particular, 40 μl of the cell suspension was spotted on each of the four disks of the FTA card, according to the manufacturer's instructions. Moreover, suspension drops were flushed in a continuous spiral way from the centre to the edge of the discs to homogeneously distribute cells on the disc area. Before cryopreservation and spotting on the cards, the cell count and viability (live, dead and total cells) were then assessed by measuring 10 μl of each suspension, stained with 10 μl of trypan blue, using a Countess™ Automated Cell Counter (Life Technologies Corporation, Carlsbad, CA, USA, cat. n. C10227).[30] The total amount of cells (cryopreserved or FTA card spotted) was neither less than 1×10^3 nor more than 1×10^7 cells in all the cases.

Cards were then dried at room temperature and stored in single, aluminum-covered, multi-barrier sealed pouches (Whatman GE Healthcare Ltd, UK, cat. n. WB100036). Pouches protect FTA cards from environmental contamination, and the tamper-evident seal maintains the sample security. Pouches are also resealable and allow multiple accesses to the cards. FC assessment was performed using the following basic combinations of phycoerythrin (PE), perdin-chlorophyll protein (PERCP) and fluorescein isothiocyanate (FITC) antibodies: CD2, CD3, CD4/8, CD2/3/7, CD5/10/19, CD19/κ/λ, FMC7/CD23/CD19, CD38/56/19. Antibodies were purchased from Becton Dickinson (San José, CA, USA), except for bcl-2, which was purchased from Pharmingen (Becton Dickinson Italia, Milan, Italy). The technical and diagnostic procedures have been previously described.[4,21,22,29] According to cytological and phenotypic data, cases were classified as non-Hodgkin lymphoma (NHL), myeloma, benign reactive hyperplasia (BRH) and inconclusive cases. NHL cases had a monomorphous cytological pattern and/or definitive cytological atypia. NHL-FC showed light chain restriction and/or with specific phenotypes such as CD5/19 co-expression, >50% CD10 expression in the gated cells or lack of one of the CD2/3/7 T-cell surface antigens. Myeloma cases were diagnosed in the presence of plasma cell proliferation and FC light chain restriction. BRH cases had polymorphous cytological features and FC polyclonal expression of light chains. Inconclusive cases had monomorphous or polymorphous smears in which FC did not express light chains or lacked light chain restriction and/or the specific phenotypes reported above. Cytologically diagnostic criteria for NHL, myeloma, BRH and inconclusive cases have been described elsewhere.[31,32] Cases of suppurative or granulomatous processes or Hodgkin lymphoma were not included in the present study. When the diagnosis of NHL was achieved, classification was performed, when possible, by cytological features and specific phenotypes.[33–35] FNC/FC diagnoses were assessed using histological controls in 80 cases of NHL and 11 inconclusive cases. The remaining 50 cases were controlled by clinical follow-up and by bone marrow biopsy and monoclonal serum picks in the four myelomas. Cytological and FC data are summarized in Table 1.

DNA isolation from FNC cells on FTA cards

DNA extraction and PCR were performed at different times ranging from 2 years to 1 month after the FNC collection. Two punches from the sample area on FTA cards were punched out using the Harris Micro-Punches, 3.0 mm diameter, with Cutting Mat (Whatman GE Healthcare Ltd, UK, cat. n. WB100038), according to the manufacturer's

Table 1. Cytological and flow cytometry clonality assessment of the present series

FNC/FC diagnoses	#	Cytological criteria N.(%)	Light chains expression N. (%)	Specific phenotypes N. (%)	Histological and/or follow-up concordance N. (%)
B-NHL	73	11 (15.1)	54 (74.0)	8 (11.0)	73 (100)
T-NHL	5	0 (0.0)	0 (0.0)	5 (100)	5 (100)
Myeloma	4	2 (50.0)	2 (50.0)	0 (0.0)	4 (100)
BRH	46	8 (17.4)	38 (82.6)	0 (0.0)	46 (100)
Inconclusive	11	11 (100)	0 (0.0)	0 (0.0)	(10 BRH, 1 T-NHL)
Inadequate	1	–	–	–	(1 B-NHL)
Total	140	32 (22.9)	94 (67,1)	13 (9,3)	140 (100)

B-NHL, B-cell non-Hodgkin lymphoma; T-NHL, T-cell non-Hodgkin lymphoma; BRH, Benign reactive hyperplasia.

instructions (Figure 1). The two 3-mm punches obtained were placed into a sterile 1.5-ml tube and used for DNA extraction by the QIAamp® DNA Micro Kit (Qiagen, Valencia, CA, USA cat. n. 56304) according to the manufacturer's protocol for DNA isolation from dried blood spots, as described elsewhere.[30] The procedure included the selective binding of DNA to a silica-based membrane of the QIAamp MinElute column, after the lyses of the samples under highly denaturing conditions at 56 °C for 1 hour, by vortexing every 10 minutes, with 20 µl of proteinase K and 180 µl of ATL Buffer. Before loading the column, an additional incubation of 10 minutes was carried out at 70 °C in the presence of 200 µl of AL Buffer, which contains guanidine hydrochloride. Samples were vortexed every 3 minutes during incubation. Lysates were drawn through by centrifugation at 8000 rpm for 1 minute, so that proteins and other contaminants that can inhibit PCR and other downstream enzymatic reactions were eluted. For the cleanup of genomic DNA, the conditions for its binding to the silica-gel-membrane were adjusted by adding 500 µl of AW1 Buffer (containing ethanol and guanidine hydrochloride) and 500 µl of AW2 Buffer (containing ethanol). The loaded column was then centrifuged at 8000 rpm for 1 minute, and elutes were discarded. DNA columns were further centrifuged at 13 200 rpm for 3 minutes to eliminate ethanol residues. DNA was then eluted from the QIAamp MinElute column using 50 µl of AE Buffer, using centrifugation for 1 minute at 13 200 rpm, after 1-minute incubation at room temperature. The obtained DNA was stored at −20 °C until its use. The DNA concentration and optical density (OD) ratio were determined by the NanoDrop 1000 V 3.7.1 Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA) and 5 µl of eluted DNA was used to test the quality by TAE 1% agarose gel electrophoresis analysis.

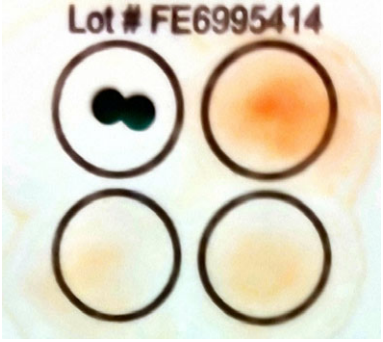

Figure 1. FTA card with spotted fine needle cytology (FNC) cells. Note the two punches used for DNA extraction in the upper left disk.

DNA isolation from FNC cryopreserved cells

DNA extraction and PCR were performed at different times ranging from 2 years to 1 month after the FNC collection. DNA was isolated from the cryopreserved cell suspensions using the QIAamp® DNA Micro Kit (Qiagen, Valencia, CA, USA cat. n. 56304), as indicated by the manufacturer for DNA isolation from small volume samples, as described elsewhere.[30] Five microliters of purified DNA was used to determine the concentration and OD ratio using a NanoDrop 1000 V 3.7.1 Spectrophotometer (Thermo Fisher Scientific) and another 5 µl was used to test DNA quality by TAE 1% agarose gel electrophoresis analysis.

Total amount of genetic material on FTA cards

To assess the total amount of genetic material contained in the FTA cards, in 10 cases (5 NHL and 5 BRH) all the FTA cards 4 discs were processed, and all the DNA was extracted to be compared with those from corresponding cryopreserved cells. For these 10 cases the number of cells spotted on the FTA cards was the same as the corresponding cryopreserved ones (Table 3). Extractions were performed using a QIAamp® DNA Micro Kit (Qiagen, Valencia, CA, USA cat. n. 56304) according to the manufacturer's protocol, as above described, respectively. DNA concentration and the OD ratio from both the sample types were determined by NanoDrop 1000 V 3.7.1 Spectrophotometer (Thermo Fisher Scientific Wilmington, DE, USA) and by TAE 1% agarose gel electrophoresis analysis. Paired FTA cards and cryopreserved samples were then amplified for exon 14 of the JAK2 gene to reveal the DNA degradation rate if any.[30,36] JAK2 PCR was carried out with 0.5 µM of each forward and reverse primers[36] in a reaction final volume of 50 µl, including 1× GoTaq(R) Green Master Mix (2×; Promega, Madison, WI, USA, cat. n. M7123). PCR reactions were performed with a Veriti® Thermocycler (Applied Biosystems, Carlsbad, California, USA) according to the following PCR Temperature profile: denaturation for 7 minutes at 95 °C, then 35 cycles (45 seconds at 94 °C, 1 minute at 58 °C and 1 minute at 72 °C for each cycle) and a final extension at 72 °C for 10 minutes. PCR products were then verified by TBE 2% agarose gel

electrophoresis analysis. Samples were always run in duplicate to ensure the PCR reproducibility.

IGHK/TCRBG PCR assessment

The BIOMED-2 multiplex PCR assays, including IGH (n = 3+2 tubes; for $V_{FR1\text{-}2\text{-}3}$-J and D-J gene rearrangements, respectively), IGK (n = 2 tubes; for V-J and V-Kde + Intr-Kde, respectively), TCRB (n = 2+1 tubes; for V-J and D-J gene rearrangements, respectively) and TCRG (n = 2 tubes), were performed as home-brew multiplexes according to van Dongen JJ *et al.* (2003) standardised protocols and primers.[15] IGHK and TCRBG PCR were performed using GoTaq® Green Master Mix (Promega, Madison, WI, USA cat n. M7123), as indicated by the manufacturer, with 0.4 μM of each primer; PCR reactions were performed with a Veriti® Thermocycler (Applied Biosystems, Carlsbad, California, USA). For each case, 100 ng of DNA of all the samples plus one negative control (no DNA in PCR reaction) was used for the IG/TCR-PCR analyses. The IGHK/TCRBG PCR products were evaluated by heteroduplex analysis (HD), as previously described.[15] Every sample was analysed twice (independent PCR amplifications from the same isolated DNA) via heteroduplex analysis and was run in duplicate to ensure the reproducibility. The IGHK/TCRBG clonality status was defined according to the EuroClonality/BIOMED-2 guidelines;[6] in particular, monoclonality was defined as the presence of one or two discrete and reproducible band(s) of attended weight for the following tubes: 310–360 bp for IGH tube A, 250–295 bp for IGH tube B, 100–170 bp for IGH tube C, 110–290 bp for IGH tube D, 100–130 bp for IGH tube E, 120–160 bp or 190–210 bp or 260–300 bp for IGK tube A, 210–250 bp or 270–300 bp or 350–390 bp for IGK tube B, 240–285 bp for TCRB tube A, 240–285 bp for TCRB tube B, 170–210 bp or 285–325 bp for TCRB tube C, 145–255 bp for TCRG tube A and 80-220 bp for TCRG tube B.[15] Polyclonality was defined as the smear on HD analysis; pseudoclonality was defined as the presence of one or two discrete un-reproducible bands or as the discrete and un-reproducible multiple (>3) bands.[6] To check the DNA quality and applicability, the Gene Control multiplex PCR was used resulting in a ladder of five fragments of 100, 200, 300, 400 and 600 bp. These fragments were obtained by amplifying the following genes: human thromboxane synthase gene (TBXAS1, exon 9, 100 bp), human recombination activating gene (RAG1, exon 2, 200 bp), human promyelocytic leukemia zinc-finger gene (PLZF, exon 1, 300 bp) and the human AF4 gene (exon 3, 600 bp; exon 11, 400 bp).[15]

In the case of the absence of the IGHK/TCRBG and CG band(s) without a polyclonal background, 50 ng of DNA from the FTA cards and cryopreserved cells were used to amplify exon 14 of the JAK2 gene to evaluate the DNA integrity, as above described. According to the IGHK/TCRBG PCR data, the monoclonal cases were considered representative of NHL and/or myeloma, and the polyclonal and pseudoclonal cases were considered representative of BRH. The IGHK/TCRBG PCR clonality testing was then verified by histological controls and follow-up.

Statistical analysis

Student's t-test for paired data, with a significance level set at 5%, was used for each diagnostic group to evaluate any statistically significant differences in corresponding median values of DNA concentration and yield and quality obtained by cryopreserved and FTA cards stored cells. The same test, for unpaired data, was performed between the medians of NHL versus myeloma, NHL versus BRH, BRH versus myeloma to evaluate whether possible differences were pathology- or method-related. Basic data evaluation were then performed to assess the sensibility, specificity, predictive values of positivity and negativity of FNC/FC and IGHK/TCRBG PCR on FTA cards in the discrimination of the three diagnostic groups; the FNC/FC inconclusive diagnoses were not included in the statistical evaluation. Conversely, possible IGHK or TCRBG polyclonal cases, subsequently proven to be B-cell or T-cell NHL respectively, were considered false negatives. IGHK or TCRBG monoclonal cases, subsequently proven to be BRH, were considered false-positives and labeled pseudoclonal.

Results

Fine needle cytology and flow cytometry

According to the above-mentioned cytological and FC criteria, 78 cases were diagnosed as NHL, four as myeloma, 46 cases as BRH, 11 as inconclusive cases and one as inadequate (Table 1); a further NHL classification was performed in 64 cases. Classification details were not reported because there was no relation with the aim of the study. Cytological and FC

diagnoses were compared with the subsequent histological and follow-up controls and showed 79 NHL (74 B-cell and 5 T-cell), four myeloma and 46 BRH proven cases. Eleven inconclusive FNC/FC cases were subsequently diagnosed as BRH (10 cases) and T-NHL (1 case). Clinical, cytological, FC, IG/TCR and controls of 11 inconclusive cases in the present series are summarized in Table 2.

DNA concentration and quality of cryopreserved and FTA cards stored cells

The number of cells utilized for DNA extraction ranged from 3×10^3 to 1×10^7 with a median of 4×10^6. DNA obtained from two FTA card punches of the 80 NHL cases ranged from 4.50 to 16.60 ng/μl, with a median of 12.20 ng/μl and the OD median ratio of 1.75 (range: 1.60–2.01). The agarose gel analysis confirmed the spectrophotometer data: a visible band of high molecular weight DNA was observed in all cases. In myeloma cases, the DNA median concentration was 8.85 ng/μl (range: 6.80–10.90 ng/μl) and the OD median ratio was of 1.95 (range: 1.88–2.01). DNA extracted from BRH cases ranged from 1.00 to 17.70 ng/μl, with a median of 11.10 ng/μl; the OD median ratio was 1.81 (range: 1.52–2.16). As far as the cryopreserved cell suspensions, the DNA median concentration was 22.80 ng/μl (range: 11.10–45.40 ng/μl) in NHL, 29.05 ng/μl (range: 28.70–29.40 ng/μl) in myeloma and 24.90 ng/μl (range: 8.40–38.70 ng/μl) in BRH. The OD median ratio was 1.72 (range: 1.58–2.01) in NHL, 1.75 (range: 1.73–1.77) in myeloma and 1.75 (range: 1.60–2.01) in BRH.

Total amount of genetic material on FTA cards

The DNA extraction from 10 cases (five NHL and five BRH), in which the whole FTA card four discs were processed, produced a DNA yield similar, in logarithmic terms, to those obtained by corresponding cryopreserved ones (Table 3). The median number of cells of these 10 cases spotted on an FTA card and corresponding cryopreserved ones was of 7×10^6 ranging from 4×10^6 to 11.5×10^6 cells. In fact, the DNA median concentration was 39.25 ng/μl (range: 19.10–99.80 ng/μl) with an OD median ratio of 1.81 (range: 1.60–2.01) from cryopreserved cells and 40.00 ng/μl (range: 17.60–89.20 ng/μl) with a OD median ratio of 1.74 (range: 1.58–1.89) from FTA cards. In the five NHL, the DNA median concentration was 43.30 ng/μl (range: 35.10–74.50 ng/μl) with an OD median ratio of 1.83 (range: 1.65–2.01) for cryopreserved cells and 48.70 ng/μl (range: 24.90–62.80 ng/μl) with an OD median ratio of 1.73 (range: 1.72–1.89) for FTA cards. In the five BRH, the DNA median concentration was 48.35 ng/μl (range: 19.10–99.80 ng/μl) with an OD median ratio of 1.74 (range: 1.60–1.93) for cryopreserved cells and 41.10 ng/μl (range: 17.60–89.20 ng/μl) with OD median ratio of 1.74 (range: 1.58–1.77) for FTA cards. Whereas OD estimations were comparable between paired FTA card and cryopreserved samples, a 500-bp band of the JAK2 gene was amplified in all the 10 samples assessing the lack of DNA degradation. In particular, the expected 500 bp JAK2 band showed similar intensity, on an agarose gel, for FTA cards and cryopreserved samples.

IGHK/TCRBG PCR assessment

The IGHK/TCRBG clonal status was the same when observed on cryopreserved and FTA card samples (Figures 2 and 3), proving the reliability of the clonality test on FTA stored cells by the home-brew multiplex BIOMED-2 based PCR assays. The Heteroduplex analysis (HD) identified IG monoclonality in 77 out of 140 cases (55%) and TCR monoclonality in six out of 140 cases (4.3%) (Table 4). Namely, IGH monoclonality was detected in 71 out of the proven 74 B-NHL (96%), in all myeloma cases (100%) and three out of 53 BRH cases (5%). IGH polyclonality was detected in eight out of 80 NHL cases (10%) and in 53 out of 56 BRH cases (95%) (Table 4, Figure 2). IGK monoclonality was detected in 66 out of 74 B-NHL (89%), in all the myeloma cases (100%) and one out of the 53 BRH cases (2%). IGK polyclonality was detected in 11 out of 80 NHL cases (14%) and in 55 out of 56 BRH cases (98%) (Table 4, Figure 2). TCRB monoclonality was detected in all the 6 T-NHL cases (100%), in 3 out of the 74 B-NHL (4%) and in 5 out of the 56 BRH (9%). TCRB polyclonality was detected in 70 of the 74 B-NHL (95%), in all the myeloma (100%) and 51 out of the 56 BRH (91%) (Table 4, Figure 3). TCRG monoclonality was detected only in all 6 T-NHL cases (100%) and in 3 out of the 56 BRH (5%). TCRG polyclonality was detected in 73 of the 74 B-NHL (99%), in all the myeloma (100%) and 53 out of the 56 BRH (95%) (Table 4, Figure 3). Amplification was not observed in all PCR negative

Table 2. Clinical, cytological, FC, IG/TCR and controls of 11 inconclusive cases of the present series

Case	Age (yrs.)	Gender	Site	Clinical data	Cytology	FNC/FC	κ/λ ratio	IG/TCR PCR	Histology/follow-up	Final diagnosis
1	75	M	Axillary sn	Previous PTL	Polymorphous pattern	CD5+, CD19+, CD2/3/7+	-/-	Monoclonal TCRB tube B and TCRG tube B	CD2+, CD3+, CD43+, CD45+ PTL	T-NHL
2	35	F	Cervical dx	–	Granulomatous pattern	CD5+, CD19+, CD2/3/7+	ne	Polyclonal IG/TCR	Follow-up negative	BRH
3	70	F	Supra-clavicular sn	–	Follicular-centre cell-rich	CD5+, CD19+, CD10+	nv	Polyclonal IG/TCR	Follow-up negative	BRH
4	62	F	Cervical sn	–	Follicular-centre cell-rich	CD5+, CD19+, CD10+	nv	Monoclonal IGK tube A	Histology negative	BRH
5	40	F	Sub-mandibular sn	–	Follicular-centre cell-rich	CD5+, CD19+, CD10+	nv	Polyclonal IG/TCR	Follow-up negative	BRH
6	75	F	Sub-mandibular dx	–	Granulomatous pattern	CD5+, CD19+, CD2/3/7+	ne	Polyclonal IG/TCR	Follow-up negative	BRH
7	55	M	Inguinal sn	–	Follicular-centre cell-rich	CD5+, CD19+, CD10+	nv	Polyclonal IG/TCR	Follow-up negative	BRH
8	47	M	Axillary dx	Lupus	Follicular-centre cell-rich	CD5+, CD19+, CD10+	ne	Polyclonal IG/TCR	Follow-up negative	BRH
9	44	F	Cervical dx	–	Granulomatous pattern	CD5+, CD19+, CD2/3/7+	nv	Polyclonal IG/TCR	Histology negative	BRH
10	58	F	Axillary sn	–	Follicular-centre cell-rich	CD5+, CD19+, CD10+	nv	Polyclonal IG/TCR	Follow-up negative	BRH
11	68	M	Inguinal dx	–	Follicular-centre cell-rich	CD5+, CD19+, CD10+	ne	Polyclonal IG/TCR	Follow-up negative	BRH

T-NHL, T-cell non-Hodgkin lymphoma; BRH, Benign reactive hyperplasia; PTL, peripheral T-cell lymphoma; nv, not valuable; ne, not expressed.

Table 3. DNA yields, OD ratio and statistical analysis from 10 cases in which DNA extraction was performed from the whole FTA card four discs and corresponding cryopreserved cells

	Cryopreserved cells			FTA cards			JAK2 gene PCR	
# Cases	Starting cells N. ($\times 10^6$)	Yield (ng/μl)	OD ratio	Starting cells N. ($\times 10^6$)	Yield (ng/μl)	OD ratio	Cryopreserved cells	FTA card cells
1	6.5	35.1	1.65	6.5	31.3	1.89	Yes	Yes
2	5.0	35.2	1.83	5.0	24.9	1.72	Yes	Yes
3	7.5	43.3	2.01	7.5	48.7	1.73	Yes	Yes
4	9.5	74.5	1.85	9.5	62.8	1.86	Yes	Yes
5	8.0	65.4	1.69	8.0	52.8	1.72	Yes	Yes
6	4.0	19.1	1.63	4.0	17.6	1.58	Yes	Yes
7	11.5	99.8	1.60	11.5	89.2	1.74	Yes	Yes
8	5.5	31.3	1.89	5.5	29.4	1.77	Yes	Yes
9	5.5	26.0	1.80	5.5	27.1	1.75	Yes	Yes
10	8.5	79.7	1.93	8.5	54.4	1.76	Yes	Yes

	Cryopreserved cells		FTA cards cells	
	Yield (ng/μl)	OD ratio	Yield (ng/μl)	OD ratio
Median	39.25	1.81	40.00	1.74
Minimum	19.10	1.60	17.60	1.58
Maximum	99.80	2.01	89.20	1.89

Student-t test	Yield (ng/μl)	OD ratio
Cryopreserved *vs.* FTA cards paired data	0.015424	0.234465

Number cases from 1 to 5 were NHL; whereas number cases from 6 to 10 were BRH.

controls by IGH, IGK, TCRB, TCRG and Gene Control multiplex PCR. The expected bands of 100, 200, 300, 400 and 600 bp obtained by the Gene Control multiplex PCR appeared in all but one case. In the latter (a B-cell NHL), neither IGHK, nor TCRBG, nor Gene Control bands were detected because of the DNA degradation, being the 500 bp expected band of the JAK2 gene absent. Conversely, in all the other samples, the JAK2 gene was amplified and no differences, either in band intensity or in PCR robustness related to collection time, were observed.

Statistical analysis

Statistically significant differences in terms of DNA concentration were found in NHL, myeloma and BRH cases when comparing the two storage methods ($P < 0.0001$), but no statistically significant differences were found between the OD ratio in NHL ($P = 0.031$), myeloma ($P = 0.023$) and BRH ($P = 0.117$). The comparison of the same values for each storage method for NHL versus myeloma showed no differences in terms of DNA concentration ($P = 0.765$ for FTA cards and $P = 0.776$ for cryopreserved cells) and quality ($P = 0.835$ for FTA cards and $P = 0.060$ for cryopreserved cells). Similar results were obtained comparing NHL versus BRH (concentration: $P = 0.166$ for FTA cards and $P = 0.651$ for cryopreserved cells; OD ratio: $P = 0.854$ for FTA cards and $P = 0.828$ for cryopreserved cells). No statistically significant differences were found when comparing the DNA concentration ($P = 0.571$ for FTA cards and $P = 0.538$ for cryopreserved cells) and quality ($P = 0.958$ for FTA cards and $P = 0.080$ for cryopreserved cells) between BRH versus myeloma. No statistically significant differences in DNA yield ($P = 0.015$) and OD ratio ($P = 0.234$) were found in the 10 cases in which DNA was extracted from the whole FTA discs and in their corresponding cryopreserved cells (Table 3).

Statistical evaluation of FNC/FC and IGHK/TCRBG PCR data in the discrimination of NHL, myeloma, BRH and inconclusive cases, when compared to with histological/follow-up controls, showed concordance in 128 out of 140 cases (91%) (Tables 1 and 4). In

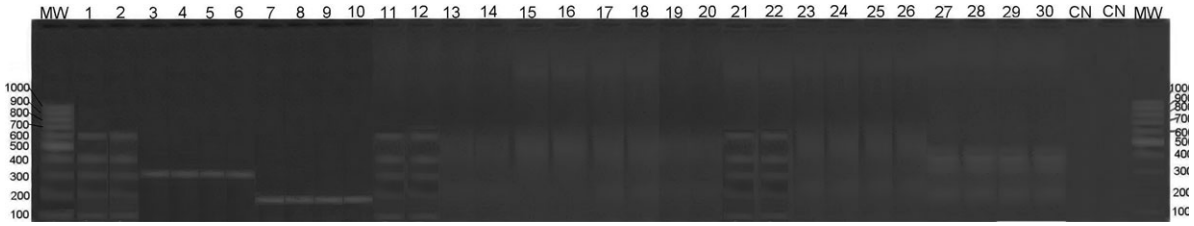

Figure 2. Heteroduplex analysis (HD) of immunoglobulin heavy and light chains (IGHK) multiplex PCR from non-Hodgkin lymphomas (NHL), myeloma and benign reactive hyperplasia (BRH) cryopreserved and FTA cards samples. *MW lane*: DNA molecular weight (100 bp AA561 diluted 1:10 Nuclear Laser Medicine S.r.l., MI, Italy); *lanes 1 and 2*: Gene Control Multiplex PCR from cryopreserved and FTA cards, respectively, B-NHL case n. 1; *lanes 3 and 4*: IGH tube A of cryopreserved monoclonal B-cell NHL case n. 1; *lanes 5 and 6*: IGH tube A of FTA cards monoclonal B-cell NHL case n. 1; *lanes 7 and 8*: IGK tube B of cryopreserved monoclonal B-cell NHL case n. 1; *lanes 9 and 10*: IGK tube B of FTA card monoclonal B-cell NHL case n. 1; *lanes 11 and 12*: Gene Control Multiplex PCR from cryopreserved and FTA cards, respectively, BRH case n. 1; *lanes 13 and 14*: IGH tube A of cryopreserved polyclonal BRH case n. 1; *lanes 15 and 16*: IGH tube A of FTA cards polyclonal BRH case n. 1; *lanes 17 and 18*: IGK tube B of cryopreserved polyclonal BRH case n. 1; *lanes 19 and 20*: IGK tube B of FTA card polyclonal BRH case n. 1; *lanes 21 and 22*: Gene Control Multiplex PCR from cryopreserved and FTA cards, respectively, T-NHL case n. 1; *lanes 23 and 24*: IGH tube A of cryopreserved polyclonal T-cell NHL case n. 1; *lanes 25 and 26*: IGH tube A of FTA cards polyclonal T-cell NHL case n. 1; *lanes 27 and 28*: IGK tube B of cryopreserved polyclonal T-cell NHL case n. 1; *lanes 29 and 30*: IGK tube B of FTA card polyclonal T-cell NHL case n. 1; *CN lanes*: negative controls (no DNA in PCR reactions).

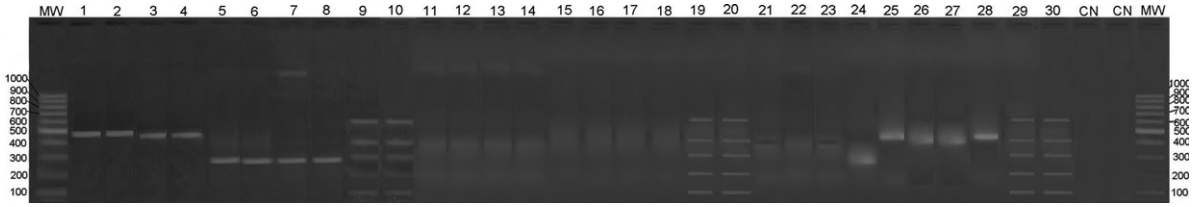

Figure 3. Heteroduplex analysis (HD) of T-Cell Receptor beta and gamma chains (TCRBG) multiplex PCR from non-Hodgkin lymphomas (NHL), myeloma and benign reactive hyperplasia (BRH) cryopreserved and FTA cards samples. *MW lane*: DNA molecular weight (100 bp AA561 diluited 1:10 Nuclear Laser Medicine S.r.l., MI, Italy); *lanes 1 and 2*: TCRB tube A of cryopreserved monoclonal T-cell NHL case n. 1; *lanes 3 and 4*: TCRB tube A of FTA cards monoclonal T-cell NHL case n. 1; *lanes 5 and 6*: TCRG tube B of cryopreserved monoclonal T-cell NHL case n. 1; *lanes 7 and 8*: TCRG tube B of FTA card monoclonal T-cell NHL case n. 1; *lanes 9 and 10*: Gene Control Multiplex PCR from cryopreserved and FTA cards, respectively, T-NHL case n. 1; *lanes 11 and 12*: TCRB tube A of cryopreserved polyclonal BRH case n. 1; *lanes 13 and 14*: TCRB tube A of FTA cards polyclonal BRH case n. 1; *lanes 15 and 16*: TCRG tube B of cryopreserved polyclonal BRH case n. 1; *lanes 17 and 18*: TCRG tube B of FTA card polyclonal BRH case n. 1; *lanes 19 and 20*: Gene Control Multiplex PCR from cryopreserved and FTA cards, respectively, BRH case n. 1; *lanes 21 and 22*: TCRB tube A of cryopreserved psuedoclonal B-cell NHL case n. 2; *lanes 23 and 24*: TCRB tube A of FTA cards pseudoclonal B-cell NHL case n. 2; *lanes 25 and 26*: TCRG tube B of cryopreserved pseudoclonal B-cell NHL case n. 2; *lanes 27 and 28*: TCRG tube B of FTA card pseudoclonal B-cell NHL case n. 2; *lanes 29 and 30*: Gene Control Multiplex PCR from cryopreserved and FTA cards, respectively, B-cell NHL case n. 2; *CN lanes*: negative controls (no DNA in PCR reactions).

particular, concordance was found in 78 out of 80 NHL (98%), in all the four myeloma cases and 46 out of 56 BRH (82%). As for the 11 inconclusive cases, subsequent histological controls revealed 10 BRH and 1 T-NHL (Tables 1, 2 and 4). IGHK PCR data were consistent with histological/follow-up controls in 138 out of 140 cases (99%); in particular, in 79 out of 80 of NHL (99%), in all myeloma (100%) and 55 out of 56 BRH (98%). TCRBG PCR data were consistent with histological/follow-up controls in 136 out of 140 cases (97%): in 76 out of 78 of B-cell malignancies (97%), in all the six T-cell NHL (100%) and 54 out of 56 BRH (96%). Finally, histological controls revealed one false-positive in the IGHK discordant case and three false-positives in TCRBG (Table 4). Sensitivity, specificity, predictive

Table 4. IGHK/TCRBG clonality assessment of the present series

FNC/FC diagnoses	#	IGH monoclonality N. (%)	IGH polyclonality N. (%)	IGK monoclonality N. (%)	IGK polyclonality N. (%)	IGHK monoclonality N. (%)	Histological and/or follow-up concordance N. (%)
B-NHL	74	71 (95.9)	2 (2.7)	66 (89.2)	5 (6.8)	73 (98.6)	73 (98.6)
T-NHL	6	0 (0.0)	6 (100)	0 (0.0)	6 (100)	0 (0,0)	6 (100)
Myeloma	4	4 (100)	0 (0.0)	4 (100)	0 (0.0)	4 (100)	4 (100)
BRH	56	3 (5.3)	53 (94.6)	1 (1.8)	55 (98.2)	1 (1.8)*	55 (98.2)
–	–	–	–	–	–	–	(1 BRH)*
Inadequate		–	–	–	–	–	(1 B-NHL)
Total	140	78 (55.7)	61 (43.6)	71 (50.7)	66 (45.1)	78 (55.7)	140 (100)

FNC/FC diagnoses	#	TCRB monoclonality N. (%)	TCRB polyclonality N. (%)	TCRG monoclonality N. (%)	TCRG polyclonality N. (%)	TCRBG monoclonality N. (%)	Histological and/or follow-up concordance N. (%)
B-NHL	74	3 (4.0)	70 (94.6)	0 (0.0)	73 (98.6)	1 (1.3)*	72 (97.3)
T-NHL	6	6 (100)	0 (0.0)	6 (100)	0 (0.0)	6 (100)	6 (100)
Myeloma	4	0 (0.0)	4 (100)	0 (0.0)	4 (100)	0 (0.0)	4 (100)
BRH	56	5 (8.9)	51 (91.1)	3 (5.3)	53 (94.6)	2 (3.6)*	54 (96.4)
–	–	–	–	–	–	–	(2 BRH; 1 B-NHL) *
Inadequate		–	–	–	–	–	(1 B-NHL)
Total	140	14 (10)	125 (89.3)	9 (6.4)	130 (92.9)	9 (6.4)	140 (100)

B-NHL, B-cell non-Hodgkin lymphoma; T-NHL, T-cell non-Hodgkin lymphoma; BRH, Benign reactive hyperplasia.
*Cases that were IGHK/TCRBG monoclonal but not histologically confirmed and labelled pseudoclonal.

values of positivity and negativity calculated on the diagnostic performances of the two methods were 100% for the FNC/FC, 99%, 98%, 99% and 98% for IGHK PCR and 86%, 98%, 67% and 99% for TCRBG PCR.

Discussion

Clonality testing is an important step in the diagnosis of lymphoproliferative processes. The role of clonality testing may be even more relevant on minimal pathological samples, such as FNC and cytological samples in general. In fact, cytological samples lack the basic features typical of the histopathological ones but, at the same time, may represent the only available material to assess whether a lymphoproliferative process is benign or malignant. The management of genetic material obtained from FNC samples raises some problems related to their specific features, such as the paucity of cells, different sources, variable clinical contexts and different diagnostic requests. Moreover, technical limitations, such as a lack of specific support for cryopreserved storage, the scanty material on cell blocks or the need to sacrifice diagnostic smears, may hamper the molecular testing of FNC samples, such that it is not routinely performed. In the last decade, molecular testing has been performed on the DNA of cells spotted on the FTA cards of different tumours.[23–28,37] In the present study, comparing the DNA concentration from cryopreserved and FTA card stored cells, statistically significant differences were found in NHL ($P < 0.0001$), myeloma ($P < 0.0001$) and BRH ($P < 0.0001$). We think that these differences are because of the complete DNA extraction from the cryopreserved cells when compared to the two punches cut from each FTA card that represents <10% of the whole corresponding absorbed area (Figure 1) and these data are comparable to those of similar studies.[27,28,38] In fact, in 10 cases (five NHL and five BRH) in which the DNA was extracted from the whole four FTA discs and the corresponding cryopreserved cells, no differences were found in the DNA yield ($P = 0.015$), the OD ratio and the JAK2 band intensity (Table 3). No statistically significant differences were found in the DNA OD ratio of different sample types and entities, and these data were further confirmed by the BIOMED-2 Gene Control Multiplex PCR. Moreover, the present data suggest that there was no protein contamination, hence no

overestimation of DNA yield, which occurred in another study.[28,38] Another potential use of the FTA cards might be the IGHK/TCRBG clonality PCR tests in cases of diffuse large B-cell lymphoma (DLBCL). In these entities, it is possible the lack of FC results is due to the fragile nature of the cells and consequent cytoplasmic fragmentation. For these reasons, we also think that these properties make FTA cards suitable for more complex molecular applications, as demonstrated by da Cunha Santos *et al.*[23] and Saeig *et al.*[26] As far as the possible use of FTA cards for biobanking, the storage time did not influence the efficacy of FTA cards storage because, as reported above, the DNA extraction and PCR were performed at different times during and no differences were observed in PCR robustness in relation to the collection times. Whereas the oldest samples of our series were 2-years old, previous studies have assessed an effective DNA storing and preservation by the FTA card longer than 17 years.[24,39–42] With reference to the accuracy of FNC/FC and IGHK/TCRBG PCR, both the methods show high sensitivity, specificity, predictive values of positivity and negativity in clonality testing. Nonetheless, beyond the statistical evaluations, a high number of inconclusive cases by the FNC/FC (11 out of 140 cases) was observed (Table 2). Inconclusive cases were determined by suspect clinical and cytological features, by FC lacking specific phenotypes and light chains not expressed or not measurable. In these cases, clonality could not be determined, and further diagnostic testing was required. Conversely, IGHK/TCRBG PCR was always effective in the diagnosis of FNC/FC inconclusive cases, enhancing its diagnostic performance of FNC. The false-positive monoclonal cases (four out of 140) detected by IGHK/TCRBG PCR also needs to be explained. Indeed, the occurrence of monoclonality in reactive processes is a well-known phenomenon that may occur in autoimmune and immunodepressive syndromes or even in enhanced physiological immune responses.[6,8–10,14,15] In the false-positive cases, the IGHK and TCRBG monoclonal status appeared as discrete and reproducible bands that have been labelled psuedoclonality as the corresponding clinical and histological data excluded any neoplastic process. The same phenomenon may be observed in FC, where pseudoclonality is generally characterised by a relatively small number of monoclonal cells.[4,43] In this perspective, the simultaneous investigation of the Immunoglobulin heavy and light chains and TCR beta and gamma chains increases the specificity of the method by the identification of true monoclonal cases. For these reasons, IGHK/TCRBG PCR is the gold standard for clonality testing of lymphoproliferative processes and the FTA cards seem to be a suitable technical procedure that perfectly matches with FNC/FC. In fact, the same cell suspension can be divided to perform both

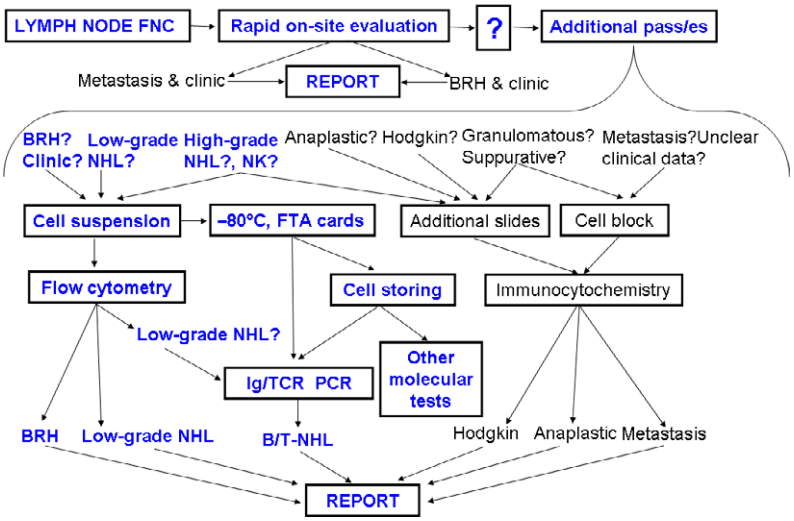

Figure 4. Proposed flow-chart for fine needle cytology (FNC) clonality assessment. Starting from FNC and immediate ROSE, the procedure continues with additional passes used to prepare buffer cell suspensions for FC and FTA cards and/or additional smears and cells suspensions to prepare cell blocks for ICC, on the basis of specific diagnostic needs.

procedures. Moreover, FTA cards are a practical and cost-effective way to store genetic material that might be conveniently used as a cytological biobank for subsequent evaluations. Therefore, we would suggest a flow-chart for the cytological assessment of clonality that starts with FNC plus immediate ROSE and continues with additional passes used to prepare buffer cell suspensions for FC and FTA cards and/or additional smears and fixed cell suspensions to prepare cell blocks for ICC, on the basis of specific diagnostic needs (Figure 4). In conclusion, the DNA yield and quality and the IGHK/TCRBG PCR results obtained by FNC and stored on FTA cards are comparable to those obtained on cryopreserved material and might represent a valid alternative to traditional DNA storage systems.

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