Molecular cytopathology: opportunities and challenges

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Demystifying molecular techniques in cytopathology practice

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Summary

The last decade was stimulating with the introduction of new molecular techniques to be applied in pathology laboratories. Accordingly, cytology was also benefited with the innovations emerged from this new era. Molecular cytopathology (MCP) can be defined as molecular studies applied on all types of cytological specimens, namely gynaecology cytology, exfoliative non-gyn cytology and fine needle aspirates. The development of a huge amount of new ancillary techniques has paralleled the emergence of clinical cytology as a major diagnostic speciality. Clinical applications of these techniques have been growing in the last decade. The widespread acceptance of liquid-based systems in gynaecological cytology is a paramount episode which re-draws the relation between cells and molecules. The stretched use of approaches, morphology and molecular biology, in HPV-induced lesions settings, e.g., revealed a potential to optimize, in one single brushed sample, diagnosis and research. Cytology samples from serous effusions, pulmonary tree, bladder urine, and aspirations, among others, are now likely to be studied by different molecular techniques for helping in diagnosis, prognosis, or even to assess therapeutic targets. In this review, we highlight the main results already published concerning the application of molecular techniques in different fields of cytopathology and discuss their application.

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- Since molecular biology results are meaningful only when interpreted with proper morphologic correlation, it is important to standardize molecular techniques at pre-analytical and analytical levels.
- In the three fields of cytology there are applications of molecular techniques that are now part of the routine assessment, such as HPV testing on cervicovaginal specimens, FISH in effusions, urine, or aspirates; for diagnosis or to assess therapeutic targets.
- As more molecular targets for therapy are identified, cytopathologists will be increasingly called upon to help identify patients who are suitable candidates for therapeutic agents that target such molecules.

Introduction

Molecular technology applied in pathology diagnostic field is undoubtedly reshaping the practice of cytopathology worldwide. A multi-institutional inquiry recently invocated an interesting discussion concerning the introduction of these methodologies to optimize cytology procedures and solving old quandaries[1]. Particular attention has been dedicated to the real utility of these new techniques and the feasibility to introduce them in cytology laboratories. The conclusions are quite exciting because most of the cytopathologists now recognize the importance of the molecular techniques as adjuncts to morphology for diagnosis. At this moment the great challenge is deciding when to adopt a new molecular test and who should perform and interpret it. Since molecular biology results are meaningful only when interpreted with proper morphologic correlation, it is important to standardize molecular techniques at pre-analytical and analytical levels. Cytology as a medical specialty is also call for changes. These include stronger working relationships between cytopathologists and bench scientists, between cytopathologists in academia and those in community hospitals and between cytopathologists and other physicians; there are also needed for new training programs that provide opportunity to understand and accelerate as we learn how better to use the tools we have and to incorporate new technologies into these processes.

There are many advantages to use cytological material over histology to perform molecular biology studies. DNA and RNA are better preserved on cytology specimens and as is easy to obtain fresh whole cells, the assessment can be immediate done. The possibility to perform genomic and proteomic studies [2,3] in small quantity of material, as for example obtained by fine-needle aspiration can minimize invasive procedures, allowing the tumour monitorization with repeating tests. Additionally, cytological samples preserved in liquid-based preservative mediums offer molecular material of high quality in comparison to those obtained from paraffin-embedded tissues. Nonogaki et al [4], showed that detection of beta-Globin, an internal control universally used to PCR assay, was detected in 100% of cervical cytological specimens against 66.7% of the corresponding biopsies. Indeed, there are some limitations to be taken in account when tissue samples are used for molecular investigation. The main limitations are related to the formalin fixative process and the paraffin inclusion. Impaired DNA and RNA preservation have been observed and this condition seriously affects its identification with the PCR assay. Likewise, the environmental conditions of storage and processing of the tissues are barely controllable, and the reproducibility of molecular assay can be seriously restricted [4]. The use of proteomics technologies have been progressively considered as a tool to monitoring protein expression in tumour cells. This approach augments the possibilities to early detect cancer recurrence and also improves cancer diagnosis recognizing proteins released by the cancer cells. Importantly, these technologies allow the use of different fresh biological samples, including tissue and cytological specimens, such as aspirates, body fluids, urine and bronchial speciments[3, 5].

The goal of this review is to present and discuss the magnitude of most available technologies and demystified the use of molecular techniques on the three fields of cytology: exfoliative gynaecological cytology, exfoliative nongynaecologic cytology and fine needle aspiration cytology (FNAC).

GYNAECOLOGICAL CYTOLOGY

The cytology of lower gynaecological tract, the notorious “Pap Test”, is certainly one of the most favoured fields of cytopathology following the introduction of new technologies. After several years demonstrating problems of poor rates of both sensitivity and unsatisfactory samples, the Pap Test was improved with the introduction of liquid-based cytology (LBC) preparations and the potential use of residual material to molecular investigation.

Importance of liquid medium

In spite of some contentious reports, LBC reduced significantly the number of unsatisfactory samples[5-7]. The sensitivity for high grade lesions of LBC in general population is quite similar to the Pap smear, but in high risk population, LBC performance is significantly superior[8]. Additionally, LBC systems have introduced qualified mediums which allows not only the cellular integrity for morphological studies, but also preserve proteins and DNA/RNA contents [9-11]. The maintenance of molecular characteristics of cells in LBC medium can also provide opportunity to study several genes involved in transformed exfoliated cervical cells [12,13]. This is vital because allow to study molecules in different conditions and for different purposes. Importantly, long term storage of LBC medium is also another essential property because permit to study samples years after its collection, with minor DNA/RNA damage. Actually, it was observed that HPV identification by Hybrid Capture II (HC2) procedure, e.g., was not affected by storage[14].

The importance of detecting high risk HPV

Presently, liquid medium are closely associated with daily routine of Pap Test, contributing with new paradigms of cervical cancer prevention. Primarily thought to be used in combination with cervical cytology, HPV testing is now seriously considered as first option for screen cervical HPV-induced lesions because the robustness of some recent data from large screening studies. Table 1 show brief current advantages to include a molecular test such as hybrid capture (HC2) as an option to screen general population. It should be considered that the differences of HC2 performance compared with cytology (Pap Test) could not only improve diagnoses but also be more cost-effective.

For many authors cervical cytology is now indicated as a reflex test to determine which HPV-positive women require additional follow-up or colposcopy[15]. HPV testing for primary screen option offers the advantage to create infection registries that can link test results from the same women over time, thus allowing an efficient and low-cost strategy to monitor long-term protection among vaccinated women[16]. This is remarkable because in the era of HPV-vaccine is important not only to discriminate HPV-negative from positive women but also to map the HPV subtypes in order to establish robust data about epidemiologic distribution of HPV worldwide[17]. Additionally, HPV test is feasible to be introduced in population out of the Public Health services through self-sampling collection. Recent data have been demonstrated that this tool is a very suitable alternative method for studies on HPV transmission and vaccine

trials[18]. In terms of laboratorial facilities, protocols for probe production and applied approaches to pathogen genotyping are now accessible through the use of highly discriminating probes, which could potentially be applied to the detection and characterization of HPV[19].

Table 1 show a comparison between a molecular method (Hybrid Capture) and morphology (cytology) to perform cervical cancer screening in large populations. Table 2, demonstrate a comparison of HPV detection among the several molecular techniques now available.

Markers for HPV integration and progression

There are many other potential useful options to be considerate for molecular marker-based assays, which can be summarised in the follow arms: chromosomal abnormalities, DNA adduct formation, cell cycle check points, oncogene expression/function, tumour suppressor gene expression, apoptotic markers, epigenetic regulation such as methylation and metabolic markers[20]. This is attractive because residual LBC material can be used for a number of purposes. One of the most accepted in cytology is the immunocytochemistry reaction which is interesting to investigate protein expression complementary to molecular analysis. The p16 accumulation is observed in intraepithelial lesions and cancer, and its expression is consequence of high risk HPV E7 activity. Overexpression of p16 is related with proliferation and in reality, the positive immunocytochemical reaction for cell cycle marker Ki-67 increased almost in parallel with the increasing grade of p16-positivity in cervical lesions[18]. Overexpression of p16(INK4A) is believed to be a diagnostic marker for cervical squamous and also glandular neoplastic lesions in LBC and a useful marker of cervical dyskaryosis[21,22]. The use of fragile histidine triad (FHIT) as a marker for cervical lesions is still contentious but its inactivation seems to be a later event, probably related with evolution from intraepithelial lesions to invasive carcinoma[23]. FHIT expression seems to occur in inverse way of the p16 because their DNA loss was commonly found in invasive carcinomas of the cervix[24]. For this reason, FHIT immunohistochemical expression is believed to be useful for prognoses purposes in cervical lesions when used in combination with p16[23].

HPV-transformed cells present alterations associated to cell adhesion properties, cytoskeleton and cell surface structures which lead to a cellular morphology alteration. The expression of members of cadherins super family, adhesion-associated proteins, may be altered in many tumours, and its recognition is valuable to diagnoses and prognoses intention. P-cadherin expression, e.g., was demonstrated to be highly specific marker to identify cervical malignancies [25].

Epigenetic mechanism of gene inactivation in cervical cancer has been recently reported. DNA methylation is an early alteration in carcinogenesis and is frequently found in precursor cancer lesions. Cervical exfoliated cells preserved in liquid medium are convenient to be used for aberrant promoter methylation analysis [26]. The frequency of hypermethylation is supposed to increase with increasing severity of cervical lesion. Frequency of hypermethylated genes such DAPK1, RARB, or TWIST1 is highly found (close to 80%) in invasive squamous cells carcinoma, moderately in high grade lesions, but very low in low grade lesions (5%). The most encouraged

observation is the absence of hypermethylation in normal cervix, which may be considerable as valuable marker to cervical cancer investigation[26].

Other different studies to investigate genomic alterations in cervical cancer are available as well, using metaphase comparative genomic hybridization (mCGH) and microsatellite marker analysis for the detection of loss of heterozygosity (LOH). At present, high throughput methods such as array comparative genomic hybridization (array CGH), single nucleotide polymorphism array (SNP array) and gene expression arrays are available to study genome-wide alterations. Together, these platforms permit the recognition of genomic variations at high resolution and, more than that, investigation of an association between copy number changes and expression, that can be confirmed by fluorescent in situ hybridization (FISH) [27]. Most genetic imbalances have been associated with decreased survival such as LOH of 18q or with the transition from severe dysplasia to invasive carcinoma such as gain of 3q. The progression of cervical intraepithelial neoplasia (CIN) 2/3 to cervical carcinoma includes integration of human papillomavirus (HPV) into the host genome, genomic instability, and an increase in chromosome 3q copy number. In particular, the gene coding for the RNA component of telomerase (TERC) at 3q26 has been implicated as a possible candidate gene which can be evaluated with the use of FISH technology [24].

Recently, FISH and chromogenic in situ hybridization (CISH) assays were demonstrated practical to be performed in both cytological and tissue samples. This type of methodology is not so complicate to standardize in cytology laboratories, allow concomitant morphological analysis and have multiple uses (see in non-gynaecological cytology). Until now, in cervical samples the study of 3q amplification is the most common [24] .

NON-GYNAECOLOGICAL EXFOLIATIVE CYTOLOGY

Serous effusions

In effusions, fluorescence in situ hybridization (FISH), comparative genomic hybridisation (CGH) and other molecular biology techniques have been successfully used to characterize malignant neoplastic cells and therefore, improve diagnostic accuracy. There are two clinical situations in which these techniques are useful: - for searching metastatic cells and for characterising of mesotheliomas.[28]. Until now, the wide-accepted ancillary method for serous effusion cytology is immunocytochemistry. Several antibodies has importantly improved the cytological diagnoses on serous effusions [29,30], but for maximising its efficiency, the use of a panel of markers is recommended because of the lack of specificity of many of antibodies [31]. Some examples are TTF-1, CK7 and CK20 (adenocarcinoma of the lung vs. metastasis of extrapulmonary adenocarcinoma), CDX2 (colorectal adenocarcinoma), BerEP-4 and calretinin (rare carcinoma cells in pleural effusions), among others [1,31], However, the variable mixture of tumor cells and normal cells, the presence of rare atypical cells still are a challenge for diagnosis using morphology and immunocytochemistry. In some situations, molecular analysis can be helpful on effusions. The detection of allelic loss in 16q22.1, including the locus of E-cadherin, in pleural effusions from breast cancer patients have showed that CDH1 gene can recognize tumour cells in pleural effusions when morphologic analysis is difficult[32]. Similarly, LOH evaluated by polymerase chain reaction assay was able to identify metastases in 36% of suspected cases where morphology only did not allow the diagnosis of malignancy.[33].

Lymphoma involving serous membranes

One of the most complex evaluations in serous effusion is the categorization of lymphoid-like cells in samples suspected of lymphomas. As anticipated, the differential diagnosis could include reactive lymphocytosis, early involvement by lymph-proliferative disease, small round-cell tumours, and presence of look-alike of Reed-Sternberg cells[34]. Immunocytochemistry (ICC) is helpful for these circumstances but have also some limitations regarding neoplastic cells phenotyping [35]. Flow cytometry (FCM) and ICC are both complementary and competitive. The preference to use one of them largely depends on experience with the technique, laboratory resources and expertise in the techniques since there are limitations of each technique[34]. The most important advantage of FCM is the multiparameter evaluation of single cells and the ability to work with very small samples. The recent availability of antibodies against the variable region of the TCR beta (V beta) chain allows determination of T-cell clonality by FCM which is not currently possible for IHC. The majority of the antibodies used for lymphoma characterization are generally available for both FCM and IHC, but some of them work better for FCM, such as antibodies to immunoglobulin κ and λ light chains, and some, such as cyclin D1 antibody, used for mantle cell lymphoma, works well with IHC [36]. Table 3 based on Kaleem (2006) review[36], summarize the main comparisons between ICC and

FCM and highlight the potential usefulness of cytological samples from serous effusions to precisely categorize the lymph-proliferative lesions.

In spite of the advantages of FCM, besides immunophenotyping, current molecular techniques are decisive in subtyping lymphomas, such as T-cell lymphoblastic lymphoma (that more frequently invade the serous membrane), follicular centre cell lymphoma, Burkitt-type lymphoma, marginal zone lymphoma, MALT lymphoma, and anaplastic large-cell lymphoma, among others. Individually, most of the known ancillary techniques have limitations; thereby, the use of multiple parameters is thought to overcome this barrier[36]. The use of PCR to diagnose T cell receptor genes in low-grade peripheral T cell lymphomas may be occasionally required; however, the vast majority of lymphomas are successfully immunophenotyped and assigned clonality by FCM or immunocytochemistry. The use of ZAP 70 by flow cytometry correlates with unmutated IgVH genes and more rapid disease progression in CLL/SLL, and if the assay is performed appropriately, may be substituted for PCR-based analysis of immunoglobulin genes.[1]

Molecular analysis of effusions

Although ancillary molecular methods, including FISH, CISH and LOH, have become part of the diagnostic armamentarium of the diagnostic pathology laboratory, thorough morphological analysis coupled with detailed clinical-pathological information is still pivotal for diagnosis-making. Moreover, a combination of molecular techniques can increase the accuracy of detecting malignant cells in dubious cases [2]. Summarising, most of the important results can be achieved with cytogenetics (karyotype) evaluation where numerical and structural alterations are important parameters to discriminate cancer from non-cancerous condition. Mesotheliomas, for example, harbour recurrent abnormalities on chromosome 6, such as loss of its long arm (6q -). At variance, adenocarcinomas in general and more specifically pulmonary adenocarcinomas show frequent alterations on chromosome 8, such as gains or inversions inv (8q). Numerical and structural alterations in chromosomes 1, 3, 6, 7, 8, 9, 11, 12 and 17 also have been described with frequency in metastatic effusions from breast and ovarian cancer. Coupling conventional cytology to cytogenetics increases diagnostic sensitivity to up to 85.3% [2]. In situ hybridization (ISH) is an important option to assist the analysis of serous effusion. Both, fluorescent (FISH) and chromogenic (CISH) in situ hybridization have been used to identify alterations in chromosomal sequences from certain tumours, in order to optimise the diagnosis of metastasis. FISH analysis with probes for chromosomes 7, 8, 11, 12, 17, and 18 in primary and metastatic breast carcinomas showed the same pattern of aneuploidy demonstrating the role of this technique in identifying individual aneusomic cells in suspicious samples [2]. Fiegl et al.[37] reported a statistically significant improvement of the cytological diagnostic accuracy using FISH analysis in effusions from breast cancer, lung cancer, pancreatic cancer, and in effusions from the entire group of gynaecological and gastrointestinal carcinomas. One of the promising methods for cytology as a whole is the comparative genomic hybridization (CGH) analysis which permits an outlining of DNA sequences and copy number alteration: losses, gains and amplification of genes are promptly identified. CGH analysis may provide information on the site of the primary tumours and also

distinguish cytogenetic alterations affecting oncogenes and tumour suppressor genes involved in neoplastic progression and metastasis[38]. An improved high resolution CGH method (array-based CGH) has been developed and already represents one of the most powerful techniques to recognise genetic alterations. DNA is hybridised in a glass slide chip with cDNA, bacterial artificial chromosome (BAC) or oligonucleotides DNA allowing evaluating the quantitative changes in the whole genome[2]. Nagel et al [38] addressed the potential use of CGH as a diagnostic adjunct for cytopathology practice in serous effusions. As immunohistochemical markers are not infallible for distinguishing mesotheliomas from metastatic carcinomas, we have begun a comprehensive analysis of primary breast carcinomas, ovary carcinomas and mesotheliomas by means of aCGH, in order to identify a molecular genetic signature that would allow differentiating these two tumour types. Although preliminary, these results are quite promising and may eventually help cytopathologists differentiating mesotheliomas from other metastatic malignancies[2]. Another useful application of CGH is to establish the relationship between a primary tumour and its metastatic deposits. Unlike immunoprofile, which can dramatically change from primaries to metastases, the DNA profiles are more stable. Even in those cases with pronounced genetic instability, genomic changes that are pivotal for tumour cell growth and survival are reported to be maintained all across tumour progression. Therefore, CGH and/or aCGH could be used to define whether a poorly differentiated/undifferentiated neoplastic deposit came from a known primary tumour or is a second primary. This was recently demonstrated in lung cancer by Yen et al [39]. In addition, we can envisage in the near future that CGH/aCGH method can at least be used for defining genetic regions that are preferentially gained or lost in mesotheliomas when compared to other tumour types that frequently metastasise to the serosal membranes, such as lung, breast, ovary and gastric cancers. By defining these regions, less complex methods, such as fluorescent and/or chromogenic in situ hybridisation or LOH, could be used for increasing the diagnostic accuracy.

The other advance that may play an important role in overcoming the diagnostic dilemma benign/reactive or malignant is the telomerase assay. Telomerase is a ribonucleoprotein complex that synthesizes telomeric repeats onto chromosomal ends during cell divisions, preventing incomplete chromosomal replication, nuclease degradation, and end—to-end chromosomal fusions. It is usually inactivated in most of adult somatic cells, which lead to cell senescence due to progressive chromosomal degradation. However, in a large subset of human cancers, telomerase is reactivated during neoplastic progression. Telomeric repeat amplification protocol (TRAP) assay has been applied to cytology with interesting results, mainly to help in the interpretation of atypical cells in breast FNA samples, as well as in effusion cytology [2]. So far, several studies have reported high sensitivity and specificity for telomerase activity as detected by TRAP in malignant cytological samples. Examples of the impact of telomerase activity analysis on effusions are on record, suggesting an increase in the diagnostic accuracy. Whilst sensitive and specific, telomerase activity is not as reliable as morphology. The false positive rates of TRAT assay range from 15 to 56% [2]. Recently, Shu et al.[40] described an in situ evaluation protocol for telomerase activity in 103 pleural effusions showing when combined with cytology, sensitivity and specificity of 90% and 95%, respectively. These

findings support that telomerase in situ assays may play a role in the evaluation of difficult cytological specimens, increasing the accuracy of the diagnosis, since TA is reported to be more frequently activated in malignant cells.

MN/CA9 is a cancer-related gene, frequently activated in human carcinomas its evaluation by RT-PCR assay is now an enthusiastically believed as a promising marker for routine practice [41]. In a recent study, MN/CA9 gene expression was detected in 53/59 (89.8%) of pleural effusions from cancer patients Importantly, MN/CA9 was positive in 13/18 (72.2%) of cytologically negative effusions of cancer patients and was detected in only 1/12 (8.3%) effusion from the control patients ($p < 0.01$) The sensitivity and specificity of MN/CA9 gene expression were, respectively, 89.8% and 91.7%. Although these results are very promising, further studies in large series needed to be done to confirm the utility of this marker.

The number of new molecules is constantly increasing, and it is reasonable to use a panel of molecules to explore in more detail all the possibilities within the effusion sample[42]. The RT-PCR is suitable for this purpose and the combination of some markers was demonstrated to be highly specific to identify malignant cells. However, the commonly used ICC marker such as carcinoembryonic antigen, epithelial cell adhesion molecule, E-cadherin, mammaglobin, mucin 1 and its isoforms MUC1/REP, MUC1/Y, and MUC1/Z, calretinin, and Wilms' tumour 1 can precisely diagnose malignancy in several conditions. Depending on the marker specificity, some of them are also capable to identify some tumours; mammaglobin, e.g., is mainly expressed in effusions from breast carcinoma reaching almost 100% of specificity[43].

Markers of therapeutic response in effusions

Recently, several studies in the field of cytology effusions have been done concerning not only diagnosis, but also prognosis and therapeutic response. NAC-1, a member of the bric-a-brac tram track broad complex/poxvirus and zinc domain family, is over-expressed in ovarian serous carcinoma. NAC-1 protein expression was found more frequent in recurrent than primary tumours[44] and the nuclear NAC-1 expression intensity is significantly higher in specimens obtained after the administration of chemotherapy been correlated with shorter progression-free survival. Moreover, NAC-1 expression is higher in ovarian carcinoma cells in effusions compared with their solid tumour counterparts. NAC-1 is believed to be up-regulated in tumour cells after chemotherapy, suggesting a role for this protein in tumour progression and in the development of chemotherapy resistance in ovarian cancer[44].

Immunoblotting method can be also adequately performed in serous effusion examination for the identification of inhibitor-of-apoptosis proteins (IAP)[45]. These proteins are related to survival and chemoresistance. Three out the eight known IAPs were lately investigated in mesotheliomas using immunoblotting and IHC: X-linked IAP (XIAP), survivin, and livin[45]. Interestingly, XIAP and survivin, but not livin, are frequently expressed in malignant mesotheliomas. Furthermore, nuclear expression of survivin is reduced in effusions as compared with solid lesions concomitantly with reduced

proliferation (assessed with Ki-67 score). XIAP was demonstrated, as well, to be up-regulated in mesothelioma effusions suggesting a pro-survival role in malignant mesothelioma cells[45].

Urine and multitargeted fluorescence in situ hybridisation

The cytological investigation of urine sample is very useful not only for diagnostic purpose but also to monitoring patients. Voided cells can be easily obtained and isolated from urine and analysed by a multitarget FISH technique using hybridization probes to chromosomes 3, 7, 9p21 and 17. The multitarget FISH has a well-recognised and important role in the management of bladder cancer[1,46-49]. This technique is standardized now and it is easily applicable to routine material. Currently FISH is used in conjunction with conventional cytology and is available to clinical use. The UroVysion® product (UroVysion, Vysis/Abbott, Downers Grove, IL) is common used in different laboratories and. three of the four FISH probes reveal duplications of chromosomes 3, 7 and 17, and the fourth probe (LSI 9p21-SpectrumGold) by its absence indicates loss of band 9p21. These findings in exfoliated urine cells are associated with transitional cell carcinoma and dysplasia, but not in reactive processes or polyoma virus infection [1]. Additionally, FISH method can identify recurrence earlier than the other methods.

Bronchial specimens and multicolour fluorescence in situ hybridization

The also called interphase multicolour fluorescence in situ hybridization (IM-FISH) is indeed a promising tool for improving cancer diagnosis as a whole, because it can directly visualize multiple changes in chromosomes and gene copy number[49]. However, multiple FISH has a low sensitivity in detecting cancer cells because presently, few targets can be detected simultaneously by current commercially available IM-FISH protocols[49]. To overcome this constraint, specific genomic probes for the genes related to lung cancer were recently developed. Multitarget FISH containing locus specific probes to chromosomes 6 and to the 5p15, 8q24 (c-myc gene) and 7p12 (EGFR gene) have been used to detect cancer cells in bronchial brushings and washings. The results showed that FISH is significantly more sensitive than conventional cytology for detecting lung cancer in bronchoscopically obtained bronchial brushing specimens and FISH detected tumours at earlier stages and detected a higher proportion of peripheral tumours [50]. However, additional studies are needed to determine if the higher sensitivity of FISH has the potential to improve patient survival. Conversely, no significant differences were found in copy number gain between high-risk individuals for lung cancer development and healthy never-smokers. The gains in chromosome 6 (CEP6), 5p15, 7p12 (EGFR), and 8q24 (C-MYC), were not associated with smoking or asbestos exposure, therefore a sensitive method is still needed for the follow-up of populations at high risk of contracting lung cancer[51]. In a technical point of view, bronchial cytological specimens can be straightforwardly used for FISH preparation from ethanol spray-fixed smears. No additional slide preparation is required prior to FISH hybridisation.

The use of large combinations of probes through the multiplex FISH is improving the diagnostic capacity on cytologic material. Moreover, better probes

to detect specific translocations will be extremely useful in the characterization of soft tissue tumours and malignant lymphomas in cytologic material. New genetic information are coming from the microarray technology and specific probes can be generated and used to obtain diagnostic, prognostic and predictive information on routine material.

FINE NEEDLE ASPIRATION CYTOLOGY

Fine needle aspiration (FNA) cytology has proven its value as a minimally invasive, easy, accurate and reliable technique for the diagnosis of several types of tumours. Moreover, in the last 15 years, FNA has also been used for the assessment of prognostic and predictive factors.

Interphase cytogenetics, such as FISH or CISH and PCR-based assays can be used in routine FNA material, for diagnostic or prognostic purposes. Sarcomas and haematological tumours are two major groups where molecular techniques are applied on FNA for diagnosis. Immunophenotyping of lymphomas using samples obtained through FNA has been successfully achieved using flow cytometry and immunocytochemistry on cytospin or even in previously Papanicolaou-stained slides[52]. Specific gene rearrangements and chromosomal translocations such as those involving c-myc in Burkitt's lymphoma t(8;14): MYC-IgG, or ALK in anaplastic large cell lymphoma t(2;5)(p23;q35), or cyclin D1 in mantle cell lymphoma t(11;14)(q13;q32) among others, can be easily detected by means of FISH on Giemsa and Papanicolaou-stained slides and fusion gene products can be detected in FNA samples through RT-PCR with specific primers[52]. Moreover, monoclonal rearrangements of the immunoglobulin heavy chain gene or T-cell receptor can be identified by PCR in FNA samples. Molecular techniques, such as FISH, RT-PCR and Southern blot technique have also successfully been applied to soft tissue tumours sampling by FNA, for the identification of chromosomal translocations, fusion gene products and gene amplification, respectively. We have previously demonstrated that DNA can be successfully obtained and amplified from archival/diagnostic cytological preparations regardless of the fixation (ethanol or air-dry) and staining methods[53].

Molecular cytology and sarcomas FNA

New FISH probes designed for specific chromosomal translocations are now commercially available. The cytological diagnosis of soft tissue tumours may eventually become even more accurate, because this technique can be prospectively applied to conventional cytological preparations. In the diagnostic setting of soft tissue tumours, the use of FISH to detected the presence of "break apart" translocation of t(11;22)(q24;q12) to diagnose Ewing´s sarcoma/PNET is one of the most common use of molecular techniques for the differential diagnosis of small round blue cell tumours[54]. The synovial sarcoma translocation t(X;18) (q11;q11) is also very useful to diagnose this tumour in cases of atypical localization and morphology. Other rare sarcomas can be detected and diagnosed on cytological material with the demonstration of specific translocations, such as: alveolar rhabdomyosarcoma t (2;13) or t (1;13); myxoid/round cell liposarcoma t (12;16); extraskeletal myxoid chondrosarcoma t (9;22), among others [1, 55]

Molecular cytology and thyroid FNA

Fine needle aspiration cytology of thyroid nodules is largely admired in pathology routine as safe and efficient methodological option to accurately diagnosed thyroid nodules [56]. However, inconclusive cytological features related to the follicular lesions are far to be solved by morphology alone and immunocytochemical available markers. Evidently, molecular analysis to rule out these constraints are welcomed, e.g. in papillary thyroid carcinoma (PTC), genetic events involve RET and TRK rearrangements, and BRAF and RAS mutations, although RAS mutations are uncommon in follicular neoplasias, except in the follicular variant of PTC[57]. These genetic alterations, which are rarely overlapped in the same tumour, result in signalling abnormalities in the mitogen activated protein kinase pathway. Alternatively, genetic alterations in follicular carcinomas include PAX8-PPAR gama translocations and RAS mutations while mutations of CTNNB1 and p53 have been implicated in the development and progression of poorly differentiated and undifferentiated (anaplastic) thyroid carcinomas[57]. Additionally, germline mutations of RET are responsible for the development of heritable forms of medullary thyroid carcinoma (MTC) while somatic mutations of this oncogene are found in a significant proportion of sporadic MTCs[57].

Although most of the molecular alterations are well-known in thyroid tumours, the study of these alterations on FNA samples are still not so useful to solve the major problems in the field. RET/PTC rearrangements and BRAF mutations can be detected in thyroid FNA but they were not present in all PTCs and can help only in cases of PTCs that simulate follicular tumours on cytology [58,59].

Figure 1 exemplifies the use of PPAR gamma gene probe to identified follicular thyroid carcinoma using FISH methodology. The gene is labelled by two colours (split probe), normal gene is yellow (overlapping red and green parts). Translocation results in split of the gene and the split of the colours: there is one green and one red spot indicated that the part of PPAR gamma was translocated to another partner chromosome. PAX8-PPAR-γ rearrangements (as the result of a 2;3 translocation) have been recurrently detected in follicular carcinomas and follicular variant of papillary carcinoma (but not in conventional PTC). The consequence of such translocation, t(2;3)(q12-13;p24-25), is fusion of the DNA binding domains of the thyroid transcription factor PAX8 to domains A-F of the PPAR-γ1. The product of the fusion gene blocks the action of PPAR-γ1, an effect that might inhibit cell differentiation and stimulate cell growth. Initially it was claimed that the PAX8-PPAR-γ translocation was exclusively detected in FTC and thus could be considered as sign of malignancy. This concept was disproved in several studies that showed the presence of the translocation in a number of follicular adenomas. Oncocytic follicular tumours, including both adenomas and carcinomas, are negative. These findings underscore the fact that PAX8-PPAR-γ rearrangements cannot be used to distinguish benign and malignant follicular cell tumours [60].

Actually, gene selection and development of diagnostic and prognostic algorithms have been reported[61]. An algorithm based on the expression levels of five genes (TERT, TFF3, PPARgamma, CITED1, and EGR2) could effectively predict high-risk disease with high specificity. TERT and TFF3

expression, e.g., discriminate adenomas from carcinomas[61]. Indeed, the analysis of atypical adenomas (AFTA) and minimally invasive follicular carcinomas (MI-FTC), showed that 16 out of 17 AFTAs were classified as benign, while MI-FTCs tend to be classified as malignant[61]. Remarkably, these findings can support the development of consistent preoperative diagnostic and prognostic tests that can guide the therapeutic approach of follicular thyroid neoplasms with indeterminate cytology.

Molecular cytology and paediatric tumours

In paediatric tumours, as mentioned before, molecular techniques (FISH or RT-PCR) have been applied in the differential diagnosis of small round blue cell tumours, showing the translocations associated to Ewings´s sarcoma/PNET t(11;22)(q24;q12) [54] or, for example, associated to desmoplastic small round cell tumor t(11;22)(p13;q12) reciprocal translocation. However, our group have more than ten years of experience studying N-myc amplification and 1q deletions in FNA from neuroblastomas to assess prognosis and to drive the clinical approach[62]. The interpretation of these results associated with the FCM anaysis of the tumour cells, allow us based only in cytological material, to separate two groups: one with good prognosis (N-myc negative and aneuploid) and other with worse prognosis and poor therapeutic response (N-myc positive and diploid). Initially studied by southern-blot, the N-myc assessment was replaced by FISH technique with the great advantage of concomitant morphological evaluation. Figure 2 document a smear from FNA of a case of neuroblastoma with MYC amplification.

Molecular cytology and therapeutic targets

Now and in the future, cytopathologists will be expected to include specific prognostic and predictive information in their reports, as well as to order ancillary tests and to contribute in clinical trials with their expertise[63, 64]. Since the last decade, there have been an increasing number of new drugs which indications depends upon a pathological report. Although lymphomas, leukaemia and gastrointestinal stromal tumours (GISTs) are the most striking examples, in breast cancer this practice is also solid and there is accumulating experience with the hormone receptors and more recently with HER2 (erbB2). The use of Trastuzumab, a humanised monoclonal antibody that acts on the HER2 receptor, had improved the survival of the patients with HER2 amplification[65]. The clinical use of trastuzumab (Herceptin) requires evaluation of HER2 amplification on tumours from every potentially eligible patient. Novel therapeutics, such as Lapatinib, an oral tyrosine kinase inhibitor, which blocks both the epidermal growth factor receptor and HER2 receptor, has been also recently approved by the US-FDA[66]. FISH is currently regarded as the gold standard method for detecting HER-2 amplification. The main difficulty for adopting FISH in a clinical setting is the need for additional equipment for analysis, such as fluorescence microscopy and multiband fluorescence filters. Recently, novel technology to detect DNA probes has been developed. Chromogenic in situ hybridization (CISH) uses a simple immunocytochemistry-like peroxidase reaction, and the results can be assessed using a normal light

microscope. The assessment is fast, with good morphologic control, and the slides can be stored for long periods without loss of signal. CISH is a reliable test to assess HER2 gene copy number[67]. A very high level of concordance between CISH and FISH has been reported. In a recent study, the rate of concordance ranged from 93% in equivocal immunohistochemistry samples to 98% in HER2 immunohistochemistry-positive cases and 97% in HER2 immunohistochemistry-negative cases, with an overall concordance rate between CISH and FISH higher than 95%[68]. The limited number of commercially available gene probes has hindered the use of CISH. However, a new protocol to generate probes for CISH was recently described allowing the construction of probes mapping to any gene of interest to be applied to paraffin-embedded tissue sections, allowing correlation of morphological features with gene copy number [69] . What is the role for HER2 assessment in FNA material? In metastatic breast cancer, Trastuzumab is used as first-line therapy after previous chemotherapy failure. In spite of good concordance between HER2 status in primary tumours and lymph node metastasis, in distant metastases this concordance diminishes with time. So, the HER2 evaluation should be preferentially done in the metastatic tumour. Performing HER2 ICC studies on FNA material remains problematic, because HER2 scoring is not validated in this material. However, HER2 assessment using FISH or CISH is now possible and useful in FNA with excellent correlation with the histologic specimens [70] (Figure 3). The recent approval for Herceptin in adjuvant therapy can expand the use of ISH in aspirates obtained from the primary tumor.

In lung cancer, the management of non-small-cell lung cancer (NSCLC) has progressively changed since the introduction of the epidermal growth factor receptor (EGFR) TK selective inhibitors, which offers an interesting new opportunity for treatment. Numerous studies have been carried out to identify patients that could better benefit from these agents. From all characteristics and clinical-pathological features, molecular finding of EGFR gene somatic mutation emerged as one of the most potentially useful prognostic and predictive factors in advanced NSCLC. Recently, it was demonstrated that genomic DNA extracted from image-guided FNA and / or cell blocks of effusion fluid are sufficient for the detection of somatic EGFR mutations using nested PCR technique [71,72]. Patients with advanced NSCLC treated by gefitinib and bearing EGFR mutations have significantly better response and progression-free survival [71] Amplification of the EGFR gene is also associated with response to therapy in some cases of lung cancer. These results can be useful in the setting of triple-negative breast cancer cases that do not have a specific therapeutic target EGFR amplification was detected by CISH in 30% of our cases of metaplastic breast carcinomas (MBCs) [73]. Given that these tumours are resistant to conventional chemo-therapy and hormone therapy regimens, our findings indicate that studies are warranted to explore EGFRTK-inhibitors as potential therapeutic agents for MBCs harbouring amplifications of EGFR.

Recently, our group demonstrated the feasibility of performing ICC and molecular analysis of c-kit and PDGFRA genes in formalin-fixed and paraffin-embedded cell blocks obtained by endoscopic ultrasound-guided FNA from GISTs[74]. This procedure allows a more precise diagnosis and therapeutic decision in the routine management of patients with GISTs. Figure 4 illustrate an example of GIST mutation detected on cytological material.

Anticipating the future

It is very difficult to predict the progress that should be anticipated over the next 10 or 50 years, because cancer is such a complex problem, with hundreds of forms and diverse means of controlling it. However, with the continued aging of the population the absolute number of cancer diagnoses will very likely rise substantially in the coming decades. So, for the foreseeable future, we will need better ways to diagnose and treat cancers. In terms of therapy, from a practical standpoint for the cytopathologist, it will be mandatory to have tumour cells available from patients participating in clinical trials in order to study the molecular features that correlate with sensitivity or resistance to the cancer drug targets. The availability of tissues or cells from the patients may allow retrospectively identification of a molecular profile or surrogate marker characteristic of responding tumours, even when the demonstration of activity is limited to a small group of patients. In turn, this profile or marker could be used prospectively for patient enrolment into subsequent studies with selected patients. At the time of tumour progression, one could also consider assessment of newly acquired genetic alterations to decide on the next line of therapy.

Fine needle aspiration cytology offers a suitable alternative to biopsy in a variety of clinical settings, in which it may be useful to obtain material to study diagnostic, prognostic and predictive markers. The progress of “specific” therapies based on antibody response will certainly obligate the cytologists to actively participate in the make decision setting for patients therapeutic options. The rapport with clinicians will be more and more interactive and both professionals should be familiarised with molecular signalling pathways. The cytological changes of tumour cells should be faced as a preliminary approach of complex, but not mystic, accessible world of molecules.

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LEGEND FOR FIGURES

Figure 1. FISH with PPAR-gamma gene probe in a case of follicular thyroid carcinoma. The gene is labelled by two colours (split probe), normal gene is yellow (overlapping red and green parts). Translocation results in split of the gene and the split of the colours: there is one green and one red spot indicated that the part of PPARgamma was translocated to another partner chromosome.

Figure 2. FISH with MYCN gene (red) probe and chromosome 2 centromere probe (green) used as a control in neuroblastoma. In a) MYCN gain (1 supplementary copy per cell) is seen in the middle cell of the smear. B) showed a case with high level of amplification.

Figure 3: FISH with HER2 gene (red) and chromosome 17 centromere (green) in an aspirate of breast cancer. In a) is demonstrated a case of polysomy of chromosome 17 and in b) a real HER2 amplification.

Figure 4: A fine needle aspiration of GIST showing a smear stained by Papanicoloau technique (a). DNA was extracted from the cells and a mutation on exon 11 of c-Kit is demonstrated by sequencing. Arrow indicates substitution of a thymine by a cytosine (L576P) (b).

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TABLES

Table 1 : Comparison between a molecular method (Hybrid Capture) and morphology (cytology) to perform cervical cancer screening in large populations.

Methodological option for primary screening	**Hybrid Capture 2**	**PapTest**
Tests per day	Model 1: 384 tests day Model 2: 800 tests day Number of professionals needed: 01	50 slides/ observer recommended For **800** samples, e.g. are needed **16** cytotechnologist
Time to training	One week	Variable: one to two years
Sensitivity	≥90%	≤60%
Specificity	High for HSIL but limited for LSIL	Usually high
Predictive negative value	≥95%	≤ 50%
Predictive positive value	Largely depends on the prevalence of the lesions	Largely depends on the prevalence of the lesions
Intervals of tests	5 years	One year
Quality Control	Included in the test	Limited efficiency and high costs involved
Reproducibility	Data similar elsewhere	Highly variable
Additional tests	Residual medium allow to perform cytology, DNA, RNA and immunocytochemistry	Very limited with conventional smear.

Table 2: Comparison of HPV detection by several molecular techniques

	Signal Amplification Hybrid Capture II (HC2)	**Signal Amplification (HC2 –Special Types - HPV 16, 18, 45)**	**Target Amplification (MY09/11)**	**Target Amplificatio o (GP5+/GP6+)**	**Genotyping INNO-LIPA (SPF1/2)**	**Genotyping Linear Array System (PGMY09/11)**	**In situ Hibridization**	**Southern Blot**	**Dot blot**
Probe	Pool of RNA probes	Pool of RNA probes	Degenerated primer	Consensus primer	Consensus primer	Degenerated primer	Oligonucleotides, ssDNA, dsDNA, RNA	Labelled Oligonucleotide	Labelled Oligonucleotide
Target	DNA/RNA Hybrids	DNA/RNA Hybrids	450 bp fragment	150 bp fragment	65 bp fragment	450 pb fragment	Varies	Varies	Varies
Analytical sensitivty	25 to 75 fg	63 to 94 fg	0.1 to 100 fg	0.5 to 10 fg	0.1 to 10 fg	900-3000 copies per cell	10-20 copies per cell		
HPV genotypes detected	13	3	39	20	43	37	Isolated types	Isolated types	Isolated types
FDA approval	Yes	No	No	No	No	No	No	No	No
Commercially available Kit	Yes	Yes	Yes	No	No	Yes	No	No	No
Level of cross contamination	Low	Low	High	High	High	High	Low	Low	High
HPV DNA quantification	Semi-quantitative	Semi-quantitative	Possible, but not optimised	Possible, but not optimised	Possible, but not optimised	Qualitative	Qualitative	Qualitative	Qualitative
Presence of fHPV multiple infection	No	No	Yes	Yes	Yes	Yes	No	No	Yes
Sensitivity	High	High	High	High	High	High	Low	Low	Low
Specificity	High	High	High	High	High	High	Low	Low	Low
Lab Working	Easy	Easy	Average	Average	Average	Average to Complex	Complex	Complex	Complex
Potential for Automation	High	High	Average	Average	Average	Low	Average	Low	Low

Table 3 Comparison of flow-cytometry and immunocytochemistry performance to study limphoproliferative diseases

Information	**Flow Cytometry**	**Immunocytochemistry**
Determination of B-cell clonality by analysis of immunoglobulin κ and λ light chains	FCM performs better than IHC	-
Evaluation of co-expression profiling of antigens on the same cells, such as co-expression of CD5 and CD23, aberrant loss of antigens, and simultaneous evaluations of cytoplasmic and surface antigens	FCM performs better than IHC	-
Evaluation of nuclear antigens such as cyclin D1 and TdT	-	IHC performs better than FCM
Lymph node FNA	FCM is often diagnostic and more informative than IHC	Informative
Diagnosis of central nervous system lymphoma in the spinal fluid in patients with acquired immune deficiency syndrome	FCM is often diagnostic and more informative than IHC	Informative
Pleural, peritoneal, and pericardial lymphocytic effusions	FCM is often diagnostic and more informative than IHC	Informative
Orbital lymphoid proliferations and intraocular fluid analysis	FCM is often diagnostic and more informative than IHC	Informative

Adapted from Kaleem Z. Arch. Pathol. Lab. Med. 2006;130:1850-8

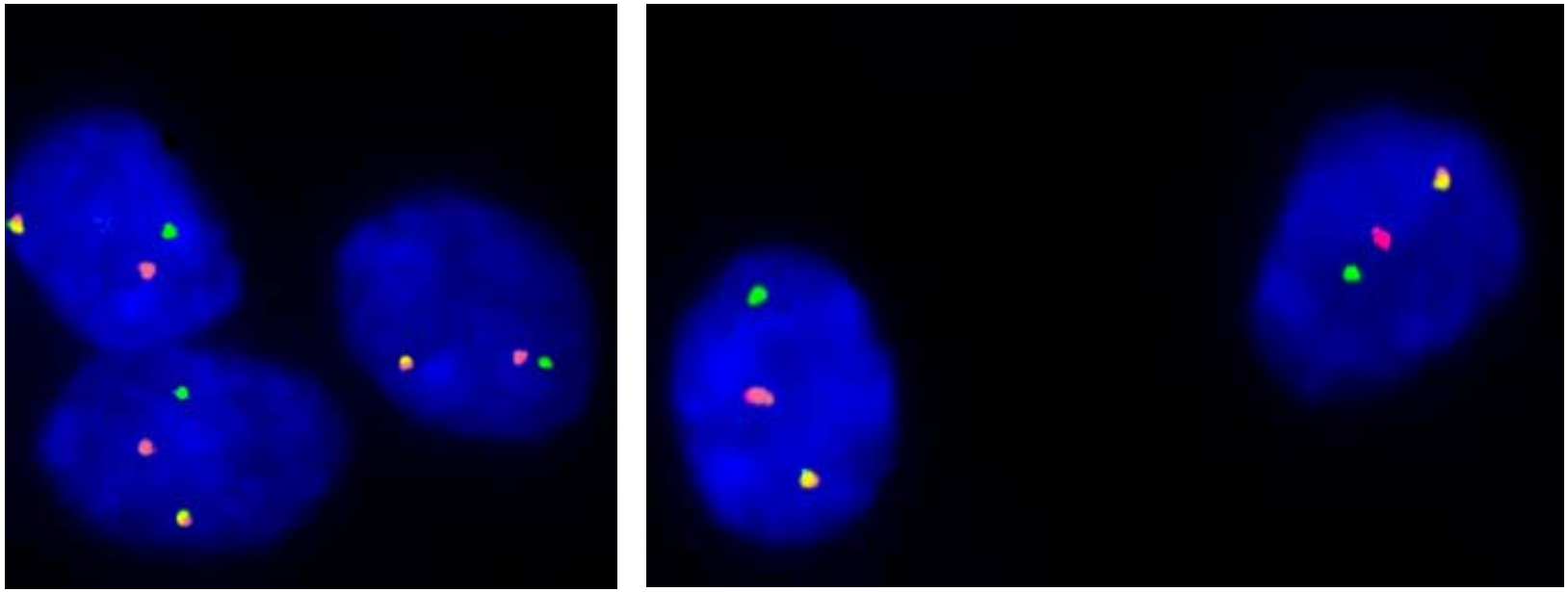
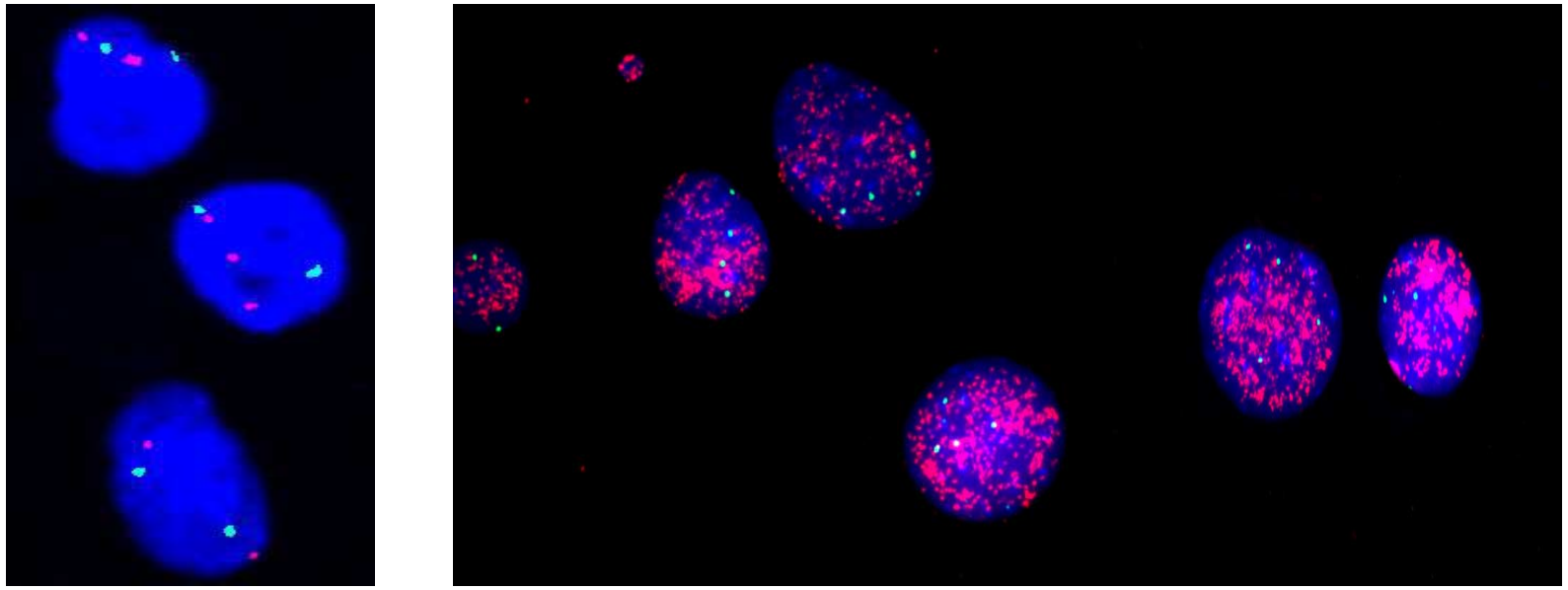
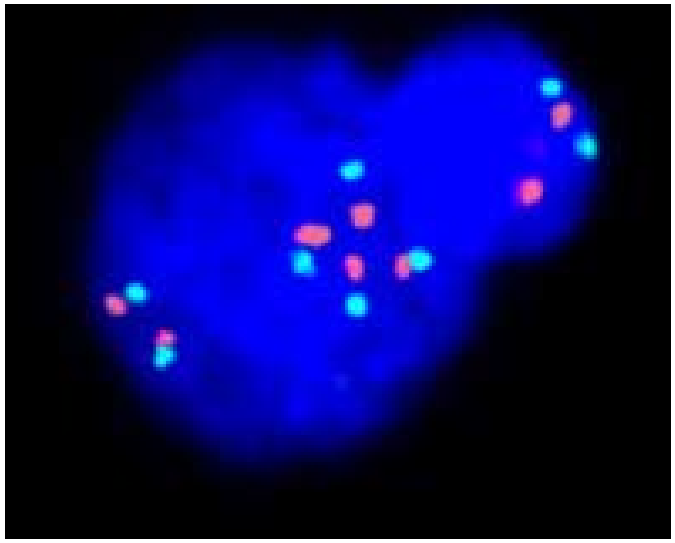


Figure 1

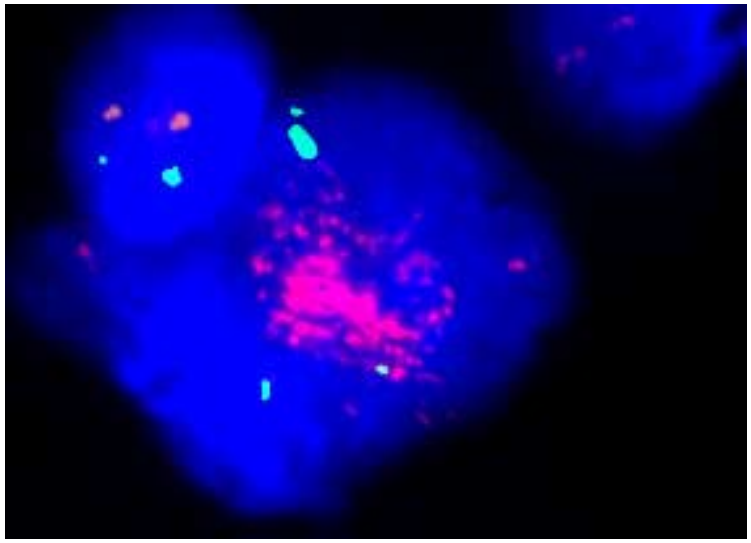


A B

Figure 2



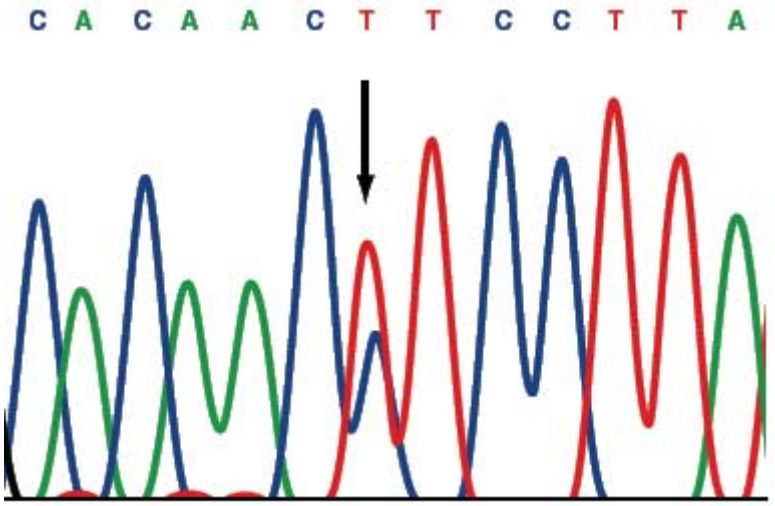
A



B

Figure 3

A



B

Figure 4