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Molecular genetic testing of uveal melanoma from routinely processed and stained cytology specimens

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Abstract

In the following study we investigated the utility of molecular genetic testing of the DNA extracted from routinely stained and processed smears from uveal melanoma (UM). Smears from five uveal melanoma cell lines and 12 primary tumors were prepared and stained with Papanicolaou and Romanowsky stains. Genotyping was carried out utilizing 14 microsatellite markers on chromosomes 3, 6 and 8. Mutational screening for alterations in *GNAQ* and *GNA11* genes was carried out by restriction fragment length polymorphism. The results were compared to those obtained through direct sequencing of frozen tumor tissues. High quality DNA was extracted from the stained slides with no difference in the efficiency of DNA extraction between the two staining techniques. The extracted DNA was of adequate quality for genotyping and mutational screening. DNA extracted from approximately 200 tumor cells is sufficient for reproducible testing of allelic imbalances and for studying the common somatic mutations in *GNAQ* and *GNA11* genes. In conclusion, we presented the feasibility of utilizing routinely stained cytology smears from UM for molecular genetic testing. The DNA obtained is of sufficient quality to carry out genotyping for markers on chromosome 3, 6 and 8, as well as screening for somatic mutations in *GNAQ* and *GNA11* genes.

Keywords

molecular genetics; eye neoplasms; uveal melanoma

1. Introduction

Molecular genetic testing of uveal melanoma (UM) can identify patients at high risk for development of aggressive disease (Damato and Coupland, 2009; Harbour, 2009; Shields et al., 2008). Loss of chromosome 3 and gain of chromosomal arm 8q has been linked to

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Conflict of interest
None declared.

Appendix. Supplementary data

Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.exer.2011.09.004.

aggressive UM. While genetic alterations in chromosomal arm 6p has been associated with less aggressive tumors (Damato and Coupland, 2009; Harbour, 2009; Prescher et al., 1996; Scholes et al., 2003). Currently molecular testing is routinely utilized as one of the most specific prognostic markers. Conventional cytogenetics, molecular cytogenetics, molecular genetics and expression based molecular testing are the different techniques performed in routine laboratories in the United States (Harbour, 2009). In Europe, multiplex ligation-dependent probe amplification (MLPA) is applied by some laboratories for routine testing in addition to the above listed techniques (Damato et al., 2009).

For tumors treated with either enucleation and excisions, any of these techniques can be utilized with variable success. However, surgically excised specimens constitute a small subset of tumors tested. Small and medium sized tumors are currently managed with brachytherapy, laser photocoagulation and proton beam irradiation (Robertson, 2003) with no tumor tissue excision. With the increasing recognition of molecular testing as an important prognostic marker there has been an increased frequency of biopsies on these tumors (Damato and Coupland, 2009; Harbour, 2009; Shields et al., 2008). Molecular techniques utilized for biopsied tumors include the above mentioned techniques with the exception of conventional cytogenetics. Irrespective of the utilized approach, the majority of ophthalmologists obtain two separate biopsies, one for cytological confirmation of the tumor and the other for molecular testing. The requirement of separate biopsies increases the potential complications during the biopsy procedure and raises concern about the source of tumor specimen due to tumor heterogeneity (Maat et al., 2007; Mensink et al., 2009; Sandinha et al., 2006). There are several additional limitations for currently utilized molecular techniques: the large cost of expression based technique (Harbour, 2009), the low sensitivity of molecular cytogenetics and its inefficiency for detection of parental isodisomy of chromosome 3 (White et al., 1998) and the high failure rate of conventional cytogenetics.

In the following report, we studied the utility of routinely processed and stained cytological preparations for molecular genetic testing of UM tumors. Testing included microsatellite based geno-typing and mutational screening using restriction fragment length polymorphism.

2. Materials and methods

2.1. Samples and cell lines

Primary UM samples were collected from patients who underwent enucleation for treatment of their tumors at the Department of Ophthalmology, The Ohio State University. A total of 12 tumors with sufficient frozen tissue for sampling were included in the study. Germline DNA was obtained from peripheral blood leucocytes. The study was carried out in accordance with an Institutional Review Board approved protocol. Table 1 summarizes the clinical data of the patients included in the study.

UM cell line MEL270 (originally established by Dr. B.R. Ksander and T.G. Murray) was kindly provided by Dr. M.J. Jager, while uveal melanoma cell lines MEL202 (originally established by Dr. B.R. Ksander and T.G. Murray), OCM3 (originally established by Dr. J. Kan-Mitchell) and 92.1 (originally established by D. M.J. Jager) were obtained from the European Searchable Tumour Cell Bank and Database. Authentication of the cell lines was described previously (Abdel-Rahman et al., 2010).

2.2. Cytological processing and staining

Cytological preparations were initially obtained from the cell lines utilizing about ten thousand cells each. For primary tumors, cytological smears were prepared from about 1–2 mm³ of frozen tissues which yielded an estimated 1×10^4 to 2×10^5 tumor cells on each

slide (Fig. 2). The slides were air dried, stored at room temperature for 24–72 h then submitted for routine cytological staining at the cytopathology laboratory, department of pathology, the Ohio State University. Two sets of slides were stained, one set with Romanowsky and the other with Papanicolaou, according to standard protocols (Allen, 1995). Papanicolaou stained slides underwent re-hydration and alcohol fixation prior to staining. Routine mounting and cover slips were applied for all slides and the smears were examined and photographed utilizing a Nikon Eclipse i50 bright-field microscope with Nikon digital sight DS-U1 5MP digital camera (Nikon, Japan).

2.3. DNA extraction

The slides were submerged overnight in Xylene to remove coverslips. The slides were then washed with two five minute changes of 100% ethanol and allowed to air dry. DNA Extraction was carried out utilizing Qiagen QIAamp DNA Microkit (Qiagen, Valenca, CA) according to the manufacturer's protocol with minimal modifications to lyse tissues from stained slides. Briefly, 180 µl of the tissue lysis buffer (buffer ATL) was added directly to each slide, mixed by pipetting and drawn into an eppendorf tube. 20 ml of proteinase K was added to each tube and samples were incubated overnight at 56 ° C. The remainder of the DNA extraction was in accordance to the manufacturer's provided protocol. The DNA integrity and quality was assessed using minigel electrophoresis and the 260/280 absorbance ratio assessed with an Epoch spectrophotometer plate reader (Biotek, Winooski, VT). Extracted DNA was stored at –20 ° C until the time of experiments.

2.4. Genotyping

Genotyping was carried out at the Nucleic Acids Shared Resource, Comprehensive Cancer Center, The Ohio State University utilizing 14 microsatellite markers on chromosomes 3, 6p and 8q according to our published protocol (Abdel-Rahman et al., 2011). Allelic imbalance factor (AIF) was determined by calculating the ratio of allele heights for both the normal (N) and tumor (T) sample, and then the tumor ratio was divided by the normal ratio: [T1:T2/ N1:N2] with N1 as the highest of the two N alleles (Cawkwell et al., 1993; Skotheim et al., 2001).

Serial DNA dilutions ranging from 60 pg to 20 ng were used to identify the lowest concentration of DNA sufficient for reproducible and stable genotyping. The serial dilution experiment was carried out on samples showing AIF less than 5 and was performed in triplicate.

2.5. GNAQ and GNA11 genes mutational screening

Mutational screening was carried out by direct sequencing of fragments obtained through PCR using DNA extracted from the frozen tissue of each tumor. Sequencing was carried out at the Nucleic Acid Shared Resources utilizing an Applied Biosystems 3730 DNA sequencer. The sequence results were read by aligning with the reference sequences provided in Genbank accession numbers NM_002072.2 (*GNAQ*) and NM_002067 (*GNA11*) utilizing the Sequencher software (Version 4.8, Gene Codes Corp, Ann Arbor, Michigan).

Mutational screening of the DNA extracted from stained smears was carried out by restriction-fragment length polymorphism (RFLP). We tested for the most common, somatic mutations in both *GNAQ* and *GNA11* genes located at codon 209 of both genes. For *GNAQ* codon 209 mutation the screening was in accordance to a previously published protocol using *Eco0109I* and *AflIII* restriction enzymes (NEB, Ipswich, MA) (Van Raamsdonk et al., 2009). Primers for codon 209 mutational screening of *GNA11* were F: 5'GGTGGGAGCCGTCTGGGAT and R: 5'GGCAGAGGGAATCAGAGGGGC. Serial

dilutions of DNA ranging from 60 pg to 6 ng were tested to identify the smallest amount of DNA needed for reproducible results. PCR was performed using a C1000 Thermal Cycler (Bio-Rad, Hercules, CA) utilizing the Qiagen Hot Start Taq master mixture with an initial incubation at 95 °C for 15 min, followed by 34 cycles of denaturation (94 °C 30 s), annealing (60 °C 45 s) and elongation (72 °C 45 s). RFLP was carried out on the obtained PCR product by overnight incubation at 37 °C with the restriction enzyme *MspAII* (NEB, Ipswich, MA).

3. Results

3.1. DNA quality and amount

The average pathlength-corrected 260/280 ratios of extracted tumor DNA from the Papanicolaou stained smears was 1.8 (range 1.6–2.0) and for Romanowsky stained smears it was 1.9 (range 1.5–2.1), indicating the high quality and purity of the extracted DNA, Table 1 supplement. This was further confirmed by gel electrophoresis that revealed slight degradation of DNA but the majority of fragments in the high molecular weight fraction (>6 kb) (Fig. 1).

The median DNA quantity obtained from Papanicolaou stained smears was 277 ng (range 114–437 ng). The median DNA quantity obtained from the Romanowsky stained smears was 224 ng (range 79–1214 ng).

3.2. Genotyping

Genotyping was performed on DNA extracted from both Romanowsky and Papanicolaou stained smears for all the 12 samples utilizing 14 microsatellite markers. PCR efficiency and genotyping of the DNA extracted from the stained smears was compared to the DNA obtained from frozen tissues from the same samples (Fig. 2). The PCR product sizes ranged from 94 to 261 base pairs. All the 14 markers were successfully amplified from all the samples. The minimum template DNA dilution that gave consistent result in all tested markers was 300 pg/reaction which is equivalent to DNA extracted from 50 cells. Lower DNA dilutions showed false positive loss of heterozygosity calls or high AIF ratios (Fig. 4).

3.3. GNAQ and GNA11 mutational testing

For each tumor the RFLP assay on DNA extracted from both Papanicolaou and Romanowsky stained slides accurately reproduced the mutational status obtained by direct sequencing of the DNA extracted from the frozen tumor tissues, Fig. 3. Template DNA concentrations of approximately 60 picogram (10 cells) were sufficient to obtain reproducible mutational assay results by RFLP.

4. Discussion

We investigated the feasibility of molecular genetic testing utilizing DNA extracted from routinely processed and stained smears of uveal melanomas. As far as we know this is the first report of the utility of this approach in molecular prognostification of UM. Our approach provides several advantages compared to the currently utilized molecular testing techniques. The most significant advantage is the utilization of the same biopsy material for both cytological assessment and molecular testing of the tumor hence eliminating the need for additional biopsies. Separate biopsies are required for almost all of the currently utilized molecular testing approaches if cytological examination is pursued. Utilization of the same specimen for both cytological assessment and molecular testing insure that the source of the tumor material used for molecular testing is representative of the tumor given the high

extent of tumor heterogeneity (Mensink et al., 2009; Sandinha et al., 2006). Additionally, single biopsy diminishes the potential complications of multiple biopsies.

The quality of the DNA obtained after processing the specimens was sufficient to carry out both genotyping and mutational screening for activating mutations in the *GNAQ* and *GNA11* genes (Van Raamsdonk et al., 2009). Preliminary studies indicate the utility of such molecular testing in stratification of the patients to MEK inhibitor based targeted therapy. We carried out microsatellite based genotyping of markers on chromosomes 3, 6 and 8 which are the most common and prognostically relevant molecular genetic markers in UM. Other markers could be included if needed.

Currently several molecular techniques are being used clinically for molecular testing of aggressive UM (Harbour, 2009). These include cytogenetics, molecular cytogenetics, MLPA and expression based molecular testing (Harbour, 2009; Onken et al.). The vast majority of molecular techniques currently utilized in clinical laboratories require separate biopsies for cytology and molecular testing. Although FISH has been reported on pre-stained cytological slides (Hammond et al., 1994; Richmond et al., 2006), we experienced very high failure rate of such technique in our preliminary studies on stained smears (data not shown). The inability of FISH to diagnose somatic isodisomy is also limiting factor for use of FISH.

There is no published data on the average number of cells obtained by fine needle aspiration biopsy in uveal melanoma. On the basis of tumor size, adequate yield for molecular genetic analysis using genotyping was obtained in 80% of small tumors 3 mm thick or less, 93% in medium tumors ranging from 3–8 mm in thickness and 75% in large tumors 8 mm thick or more (Shields et al., 2007). No cytological assessment was carried on these tumors. Expression based molecular testing was successful in 94.8% of samples even in those with inadequate cells for cytological assessment. However, it should be noted that the cytological assessments were carried out using separate biopsies (Onken et al., 2010). Based on our experience the number of cells obtained by fine needle biopsy of uveal melanoma ranges from few hundred to few thousands cells. Visualization of the tumor material to be utilized for molecular testing is important to insure the source of material used for such test.

One of the limitations of genotyping assay is that at low DNA concentrations preferential allele amplification could lead to false positive LOH pattern. A critical level of around 50 alleles per PCR, or 25 cells, has been suggested in order to obtain stable LOH results (Slebos et al., 2004). However, such estimates were obtained from DNA extracted from peripheral blood leukocytes which were processed immediately with no air drying or staining. Since our technique results in partial degradation of DNA we investigated the smallest amount of DNA that can produce stable genotyping results. We identified a concentration of 300 pg, equivalent to DNA extracted from 50 cells, as sufficient for reproducible detection of allelic imbalances. For detection of *GNAQ/GNA11* mutation status lower amount of DNA 150 pg (25 cells) are needed. In total, DNA extracted from 200 cells will be sufficient to produce stable genotyping and mutational status estimates.

We selected Papanicolaou and Romanowsky stains for our testing since they are the most widely utilized cytological stains in clinical laboratories. In our protocol we air dried the prepared smears according to the protocol carried out for routine specimens at our center. The air drying simplifies the process for smear preparation. To minimize drying artifacts for slides stained by Papanicolaou stain, slides were rehydrated then fixed in ethanol before staining according to routine protocol (Allen, 1995). Ethanol fixation of smears without airdrying is used by some laboratories for Papanicolaou staining. Such immediate fixation could improve the quality of our assay as ethanol fixation minimizes DNA degradation. However, this step could be more challenging to clinicians performing the biopsy.

Microsatellite based genotyping is currently available in the vast majority of molecular pathology laboratories. The technique is well established, robust, relatively inexpensive, can be carried out for single or small number of patients and can be easily interpreted without the necessity of complex data analysis. SNP genotyping could be a future alternative approach for molecular testing of DNA extracted from stained smears. SNP genotyping can identify both copy number alterations and loss of heterozygosity status (Onken et al., 2007). In addition, mutational screening for *GNAQ* or *GNAI1* can be incorporated in multiplex SNP platforms. Several approaches have been established for high-throughput SNP genotyping. However, the majority of these platforms are still not utilized in routine clinical settings.

Our approach could provide an important quality control step prior to molecular testing in clinical laboratories to insure the existence of malignant cells in the biopsy material to be tested. However, it should be noted that current Clinical Laboratory Improvement Amendments (CLIA) guideline requires the retention of the cytological preparation for at least five years from the time of diagnosis. This requirement could limit the utilization of cytological preparations used for routine pathological assessment for molecular testing. With the improvement of slide imaging techniques, including virtual microscope, such limitation might be minimized in the near future.

In conclusion, we presented a feasibility study on utilizing routinely stained cytology smears from uveal melanomas for molecular genetic testing to assess tumor aggressiveness. The DNA obtained is of sufficient quality to carry out genotyping for markers on chromosome 3, 6 and 8 as well as for mutational screening for somatic mutations in *GNAQ* and *GNAI1* genes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. DNA extraction from routinely stained and processed cytological smears. Smears prepared from tumors were stained for (A) Papanicolaou (P), (B) Romanowsky (R) stains, shown both at 400× magnification. (C) Gel electrophoresis of representable samples showing the quality of the DNA extracted from the stained slides. Note that the majority of samples show DNA fragments > 6 kb.

Fig. 2. Efficiency of DNA extracted from stained smears for genotyping. Genotyping results for three different microsatellite markers utilizing DNA extracted from either frozen tissue, Romanowsky or Papanicolaou stained smears. DNA extracted from stained smears showed similar results to the one extracted from frozen tissue.

Fig. 3.

Mutation screening for sequence alterations in the 209 codon of *GNAQ* and *GNAI1* genes. A) and B) showing sequence variant detected by sequencing of exon 5 of *GNAQ* and *GNAI1* genes respectively. C) and D) Representative samples showing WT and heterozygous nucleotide alteration of the 209 codon of *GNAQ* and *GNAI1* genes respectively as detected by restriction fragment length polymorphism (RFLP). P: Papanicolaou; R: Romanowsky stains. A negative control with no PCR amplification was included in the first step of RFLP assay before enzymatic digestion (data not shown).

Fig. 4. Lowest amount of DNA sufficient to reproducibly identify molecular genetic changes. A) Representative sample showing the Allele Imbalance Factor (AIF) ratios obtained using replicas of the listed DNA dilutions. B) Restriction fragment length polymorphism (RFLP) using the listed DNA dilutions. A negative control with no PCR amplification was included in the first step of RFLP assay before enzymatic digestion (data not shown).