To close this gap, we developed an assay with a short amplicon of 95 bp to enable sensitive and reliable detection of PPV DNA, even in samples of moderate DNA quality, such as DNA extracted from paraffin- or formalin-fixed tissue. Although there is a divergence of up to 15.6% in the B2L gene sequences of different PPV species, careful selection of primers and probe gave satisfactory and comparable amplification efficiency as well as a detection limit for the PPV species ORFV, BPSV, PCPV, and SPV, which was confirmed by analysis of 41 clinical samples from humans or animals infected with these PPV species.

In summary, the PPV real-time PCR assay is a rapid and useful diagnostic tool to identify PPV in clinical specimens. This may be important in both veterinary and human diagnostics in cases when differential diagnosis from other skin tropic infections, especially with OPV, is mandatory for risk assessment.

DNA from sealpoxvirus was kindly provided by Paul Becher (Institut für Virologie, Justus-Liebig Universität, Giessen, Germany).

References


Use of RNA Fluorescence In Situ Hybridization in the Prenatal Molecular Diagnosis of Myotonic Dystrophy Type I, Emanuela Bonifazi,1 Francesca Gullotta,1 Laura Vallo,1 Raniero Iraci,1 Anna Maria Nardone,2 Ercole Brunetti,3 Annalisa Botta,1 and Giuseppe Novelli1,2 1 Chairs of Human Genetics, Department of Biopathology and Diagnosing Imaging, Tor Vergata University of Rome, Rome, Italy; 2 Medical Genetics Unit, Policlinico Tor Vergata, Rome, Italy; 3 Research Centre S. Pietro, Fatebenefratelli Hospital, Rome, Italy; * address correspondence to this author at: Cattedra di Genetica Umana, Dipartimento di Biopatologia e Diagnostica per Immagini, Facoltà di Medicina e Chirurgia - Ed. E-Nord, Università di Roma “Tor Vergata”, Via Montpellier, 1, 00133 Rome, Italy; fax 39-6-2042731, e-mail emanuelabonifazi@yahoo.it

Background: Myotonic dystrophy type 1 (DM1; OMIM #160900) is an autosomal-dominant genetic disorder with multisystemic clinical features associated with a CUG expansion in the 3’ untranslated region of the DMPK gene on chromosome 19q13.3. A long-PCR protocol to detect the DM1 expansion is rapid, sensitive, and accurate, but interpretative limitations can occur when the expansion size exceeds the PCR amplification range and in cases of somatic mosaicism.

Methods: To overcome these problems, we used RNA fluorescence in situ hybridization (RNA-FISH) to study cultured cells derived from chorionic villus samples (CVS) with the DM1 mutation. The RNA-FISH method is designed to detect the distinctive DM1 cellular phenotype, characterized by the presence of nuclei with focal ribonuclear inclusions (foci) containing the DMPK untranslated region (DM1-FISH).

Results: In 4 DM1-predicted fetuses with a CUG expansion >200 CUG, varying numbers of ribonuclear inclusions were clearly visible in all cells. One case with a somatic mosaicism for the DMPK mutation showed 15% of cells with no nuclear foci. No nuclear signals were detected in all controls examined (n = 6) and in 1
Myotonic dystrophy type 1 (DM1; OMIM # 160900) is an autosomal dominant genetic multisystemic disease, with a occurrence rate of ~3 to 15 per 100 000 people in most European populations (1). Progressive muscle weakness, myotonia, cataracts, and cardiac arrhythmia (1) characterize the clinical phenotype. The DM1 gene (dystrophia myotonica protein kinase; DMPK) is localized on chromosome 19q13.3 and contains a (CTG)ₙ trinucleotide polymorphism in the 3’ untranslated portion (2-4). In normal individuals, the repeat size ranges from 3 to 37 repeats, whereas patients with DM1 have expansions of >50 repeats and, often, as many as several thousand repeats in the congenital form of the disease (4). DM1 genotypes can be divided into E1 (50–200 CTG), E2 (200–1000 CTG), and E3 (>1000 CTG) classes, with the higher number of copies associated with an aggravation of the clinical phenotype and an earlier onset of symptoms (5, 6). Expanded (CTG)ₙ repeats are unstable and usually tend to expand in successive generations, especially when transmitted through mothers (7, 8).

The somatic cell mosaicism characteristic of DM1 depends on a patient’s age, the tissues examined, and the length of the expansion (9-12), and makes the molecular diagnosis and the prognosis of the disease complex. Direct analysis by Southern blot with a DMPK-specific probe was the first method for studying the DM1 mutation (2-4). Although this procedure can be applied easily in DM1 postnatal diagnosis, it requires relatively large amounts of intact, high–molecular-weight DNA (hundreds of nanograms to micrograms), and it is time-consuming.

We currently use our long-PCR protocol (13), which involves a single genomic in vitro amplification, followed by a high concentration agarose gel electrophoresis and (CTG)ₙ oligo-specific hybridization. This protocol is rapid, sensitive, reproducible, and accurate in DM1 prenatal molecular diagnosis, but interpretative limitations can occur when only poor-quality DNA is available, the expansion size exceeds the PCR amplification range, and in the presence of a somatic mosaicism. To overcome these problems, we used the RNA fluorescence in situ hybridization (RNA-FISH) to detect the DM1 mutation in chorionic villus samples (CVS) derived from in vitro-cultured cells.

The RNA-FISH method is based on the distinctive DM1 cellular phenotype, characterized by the presence of nuclei with focal ribonuclear inclusions (foci) (14) containing the DMPK expanded transcripts. This anomalous RNA does not follow the expected pathway for nucleocytoplasmic transport, but accumulates in the nucleus sequestering proteins implicated in the alternative splicing process (15-18). Ribonuclear foci have been detected in nuclei of DM1 muscle cells, fibroblasts, and neurons (14-18), but no qualitative and quantitative data have been reported on the presence of such alteration in other cell types (e.g., trophoblast cells).

We have analyzed 6 CVS from DM1 predicted pregnancies and 6 CVS from DM1-negative predicted pregnancies as negative controls with use of our 3-step molecular diagnostic procedure: (a) A first fluorescent PCR-based sizing across (CTG)ₙ repeats is used to exclude samples with 2 normal alleles. (b) In homozygous samples, the DM1 expansion is detected and sized with the long-PCR–based protocol combined with linkage analysis. This tracks the “at risk” haplotype in the pedigree and excludes maternal contaminations. (c) Molecular cytogenetic evaluation of trophoblast samples is obtained by RNA-FISH with a (CAG)₁₀-Cy3 fluorescent probe able to visualize the DMPK expanded transcript.

In control fetuses, the DM1 mutation was excluded by PCR analysis and capillary electrophoresis on ABI Prism 310 (Applied Biosystem, Inc.), which detected 2 DMPK alleles in the normal range. In all DM1-predicted fetuses except 1, we detected expanded alleles in the range of E1-E3 classes (samples 1, 3–6). Molecular analysis of CVS from case 2 revealed the presence of 2 different and distinct mutated alleles (80 CTG and 370 CTG; Fig. 1D), indicating a somatic mosaicism for the DMPK mutation.
Table 1. Results of molecular characterization and cytogenetics evaluation by RNA-FISH analysis of CVS samples from DM1-predicted pregnancies and from controls.a

<table>
<thead>
<tr>
<th>Analyzed samples</th>
<th>CTG repeats</th>
<th>Foci, mean/cell</th>
<th>Positive cells, %</th>
<th>Parental transmission of DM1 mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>&lt;30</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Case 1</td>
<td>75</td>
<td>0</td>
<td></td>
<td>PT^a</td>
</tr>
<tr>
<td>Case 2</td>
<td>80 - 370</td>
<td>9.24</td>
<td>92</td>
<td>PT</td>
</tr>
<tr>
<td>Case 3</td>
<td>950 - 1100</td>
<td>2.94</td>
<td>100</td>
<td>MT</td>
</tr>
<tr>
<td>Case 4</td>
<td>1000 - 1300</td>
<td>3.99</td>
<td>100</td>
<td>MT</td>
</tr>
<tr>
<td>Case 5</td>
<td>450</td>
<td>5.73</td>
<td>100</td>
<td>MT</td>
</tr>
<tr>
<td>Case 6</td>
<td>650 - 790</td>
<td>5.88</td>
<td>100</td>
<td>MT</td>
</tr>
</tbody>
</table>

a Parental transmission of expanded alleles is also indicated.

b PT, paternal transmission; MT, maternal transmission.

(1). Parental origin of the DM1 mutation was determined by microsatellite analysis, which also excluded the possibility of contamination in maternal transmissions (Table 1).

RNA-FISH analysis was performed as described in the first report on the presence of nuclear foci in DM1 cells (19), with slight modifications. Cultured trophoblast cells were grown directly on cover slips, washed once in 1× phosphate-buffered saline (PBS; 100 mmol/L Na2HPO4, 20 mmol/L KH2PO4, 137 mmol/L NaCl, pH 7.4; Cambrex), and fixed for 10 min at room temperature in 4% formaldehyde (40% liquid stock purchased from Electron Microscopy Science), 100 mL/L acetic acid (Carlo Erba), 1× PBS. After 2 washes with PBS, cells were permeabilized by treatment with 700 mL/L ethanol (Carlo Erba) for at least overnight at 4 °C. Cells were rehydrated for 5 min at room temperature in 2× standard saline citrate (SSC; 300 mmol/L NaCl, 30 mmol/L sodium citrate, pH 7; Carlo Erba)–50% formaldehyde and hybridized overnight at 37 °C with (CAG)\textsubscript{10}-Cy3 oligonucleotide probe (Sigma) in Hybridization Buffer (Vysis). The hybridization of this probe to nuclear foci did not require denaturation. Cells were washed twice for 30 min at the appropriate stringency: 2× SSC–500 mL/L formamide at 37 °C. Slides were washed in 1× PBS and mounted with an antifade reagent in DAPI (0.1 mg/L; Vector), and observed with an Olympus BX61 fluorescence microscope equipped with a digital camera. Pictures were elaborated with CytoVision software (Olympus). To prevent the contamination of preparations with nucleases, gloves were worn throughout the procedures. Moreover, RNase-free or DEPC-treated water and solutions for the detection of RNA were used.

No nuclear signals were detected in all controls examined (n = 6) and in 1 DM1-positive sample (case 1) with a CTG expansion <100 copies (Table 1; Fig. 1A). In all others samples with a CTG expansion >200, various numbers of ribonuclear inclusions were clearly visible in cells (Table 1; Fig. 1B). Mutant DMPK RNAs were not localized to a particular subnuclear structure, and the number of fluorescent spots was extremely variable, depending on cell cycle status in each DM1 CVS analyzed. No direct correlation was seen between the expansion size and the mean number of foci observed at a cellular level (Table 1). Case 2 (Table 1), with a somatic mosaic at the DNA level (Fig. 1D), showed 15% of cells with no nuclear foci (Fig. 1C). This observation also indicated that, contrary to a previous report (8, 12, 20.), repeat heterogeneity in DM1-affected fetuses starts during the first trimester of gestation.

The data obtained in this study demonstrated that nuclear foci, and therefore the DM1 mutation they are caused by, can be detected efficiently on interphase nuclei of trophoblast cells by RNA-FISH. The method produced no false-positive results and detected foci in all cases examined with (CTG)n >100.

Theoretically, this protocol could also be applied for prenatal molecular diagnosis of DM2, because the (CAG)\textsubscript{10}-Cy3 probe binds the ZNF9 mutated transcript in myoblasts from patients (21). Thus, the RNA-FISH protocol proposed is a diagnostic tool specific for DM, where the pathogenesis is caused by the retention of aberrant, expanded transcripts in the nucleus.

The inclusion of RNA-FISH in the DM1 prenatal monitoring of at-risk fetuses presents several improvements to the currently used analytical procedures. In particular, the method proposed here can resolve diagnoses where data from linkage analysis of DM1 allele transmission and direct analysis of DM1 mutation are discordant (e.g., because of failure to detect the mutated alleles). These cases could be caused by the presence of somatic mosaicism, DM1 expansions too large to amplify by long PCR, or both. The definition of the exact size of the expanded allele reveals difficulties related to the molecular heterogeneity, which requires experienced observers. The proposed approach to antenatal diagnosis of DM1 makes explicit the exact nature and the distribution of the (CTG)n expansion, providing additional information about fetal genotype.

This work was supported by grants from the Italian Ministry of Health, the Italian Ministry of Education, University and Research (FIRB and COFIN), and the Istituto Superiore di Sanità.

References

Point-of-Care i-STAT Cardiac Troponin I for Assessment of Patients with Symptoms Suggestive of Acute Coronary Syndrome, Fred S. Apple,* Ranka Ler, Adrine Y. Chung, Michael J. Berger, and MaryAnn M. Murakami (Hennepin County Medical Center and University of Minnesota School of Medicine, Department of Laboratory Medicine and Pathology, Minneapolis, MN; * address correspondence to this author at: Hennepin County Medical Center, Clinical Laboratories MC P4, 701 Park Ave., Minneapolis, MN 55415; fax 612-904-4229, e-mail fred.apple@co.hennepin.mn.us)

**Background:** Few studies have investigated the role of cardiac troponin point-of-care (POC) testing for predicting adverse outcomes in acute coronary syndrome (ACS) patients. We investigated the use of a POC cTnI assay in ACS patients.

**Methods:** We studied consecutive patients (n = 367) presenting with symptoms suggestive of ACS who were admitted through the emergency department. We measured plasma cTnI with the i-STAT assay. Patients were risk-stratified based on cTnI concentrations defined by the predetermined 99th percentile reference limit for plasma (0.04 μg/L). Patients were followed for 60 days. We computed survival and event curves with the Kaplan–Meier method and compared risk stratification groups with the log-rank test.

**Results:** Acute myocardial infarction (MI) was diagnosed in 8.1% of patients. Odds ratios and 95% confidence intervals for all-cause death (ACD), MI or ACD, MI or cardiac death, and cardiac death at 60 days were all statistically significant after adjustment for age, diabetes, hypertension, and history of renal failure as follows: 2.54 (1.24–5.20), P = 0.009; 2.76 (1.37–5.58), P = 0.003; 5.98 (1.65–21.7), P = 0.008; and 2.54 (1.24–5.20), P = 0.009. Kaplan–Meier curves showed early separation between patients with increased vs. reference concentrations before 30 days for ACD, MI or ACD, and MI or cardiac death.

**Conclusion:** The i-STAT POC cTnI assay can be added to the list of assays for risk stratification.

© 2006 American Association for Clinical Chemistry

Numerous studies have evaluated cardiac troponin I (cTnI) and cardiac troponin T (cTnT) as markers for risk stratification of acute coronary syndrome (ACS) patients. Metaanalyses have demonstrated that cTnI or cTnT concentrations measured at the time of admission can be used to predict adverse outcomes (1). Increased cardiac troponins in ACS patients have been correlated with severity of coronary artery stenosis (2). Because analytical and clinical differences exist among cardiac troponin assays (3,4), cardiology (5,6) and laboratory medicine (4,7) have endorsed the need for evidence-based studies before individual assays are accepted into clinical practice. In a 2000 consensus document from the European Society of Cardiology (ESC) and the American College of Cardiology (ACC), myocardial infarction (MI) was redefined as any amount of myocardial necrosis in the presence of myocardial ischemia, as indicated by an increased cardiac troponin above the 99th percentile of a reference population (5,6). Although assay precision is important for risk stratification (8), recent data have demonstrated that assay imprecision of 10% to 25% will not significantly misclassify patients by ruling out MI (9).

Few studies have investigated the role of point-of-care (POC) testing for assessing adverse outcomes in ACS patients. One study using qualitative POC assays for cTnI and cTnT showed both assays to be independent predictors of cardiac events in ACS patients 30 days after admission (10). Although patient risk was successfully stratified with both qualitative assays, higher concentra-