Brief Communication

Atypical 22q11 microdeletions in Iranian patients with congenital conotruncal cardiac defects

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A highly characteristic feature of 22q11DS (22q11 Deletion Syndrome) is congenital heart disease (CHD), which occurs in approximately 75% of all patients.[1] Characteristics of congenital cardio vascular defects associated with DG/VCF (DiGeorge/Velocardiofacial syndrome) are termed tetralogy of Fallot including pulmonary atresia and ventricular septal defect (VSD) in the severest type, truncus arteriosus communis and interrupted aortic arch. The great majority of patients suffering from the del22q11.2 syndrome (97-98%) have a common proximal breakpoint, while the distal breakpoint can vary, and in 90% of patients it produces a 3 Mb deletion (typical deleted region [TDR]); or in 7% of patients produces a 1.5 Mb deletion. While smaller numbers have "atypical" deletions or translocations, and could present with symptoms similar to 22q11DS. Furthermore, other chromosome defects such as 4q, 10p13, 18p21, and 17p13 with features of DiGeorge anomaly have also been described.[2] The currently accepted clinical laboratory assay for 22q11DS is fluorescent in situ hybridization (FISH),[3] but this technique has some drawbacks. Fluorescent in situ hybridization is mostly efficient when the breakpoints are the same in all patients. Otherwise using FISH can produce false negative results. Special expertise and equipment, which are prerequisites of FISH cannot be found easily in most laboratories.[4] Based on these facts, we designed and set up a novel semi quantitative polymerase chain reaction (PCR) for detection of 22q11.2 microdeletions and microduplications. Multiplex ligation dependent probe amplification (MLPA) with P023 probe mix was used to validate our data.

This study was conducted from January 2007 to January 2008 in the Molecular Genetics Laboratory, Sarem Women Hospital, Tehran, Iran. Ten patients were selected based on the presence of congenital conotruncal cardiac defects (inclusion criteria). Patients or their parents signed a consent form approved by the local ethics committee. Peripheral blood of 3 normal individuals was used as normal control. The results of FISH analysis by commercial region probe TUPLE1, carried out in the Laboratory of Cytogenetics, Markaz Tebi Hospital, Tehran, Iran, were taken into consideration while comparing molecular and FISH results. Genomic DNA was extracted from fresh peripheral blood of the patients and controls using the salting out method. Five pairs of primers were designed and verified by primer3plus program to set up multiplex PCRs (D22S944-F: CAT GTG AAA GAT GCT ACT TCC, D22S944-R: ATC CCA TGC TCC TCC CCA T; D22S936-F:CAA TCT TGG CAG CCA GTT TAG, D22S936-R: CAG CAT CTT CCT GGT GGC C; D22S636-F:AAC CTT CTG ATG GCT CCT CT, D22S636-R: CAT GGA GCT GAC ACT GAG TG). Three of the primer sets amplify established sequence tag site (STS) markers. Two of these markers reside in the 3 Mb TDR (D22S944, D22S936), and one is situated outside this region (D22S636). The last marker is used as internal control. Two remaining primer sets amplify HPRT and P15567 (HPRT-F: ACG TCT TGC TCG AGA TGT GA, HPRT-R CCA GCA GGT CAG CAA AGA AT; P15567-F: GCC AGA GGA TAG GGA GTG C, P-15567-R GTG GAA GCA GTC AAA CAG AAC) and were used as known copy number controls. The P155675 is situated on the 5'flanking region of TDR. The PCR reactions were performed in 25 µl total reaction volume containing: 0.25 unit of Taq polymerase (CinaGen, Iran), 2 mM of dNTP mix (CinaGen, Iran), 1X PCR buffer (CinaGen, Iran) (P15567-HPRT coamplification) or 2X PCR buffer (D22S936-D22S636 coamplification) and 200 nM of each primer. The D22S944-D22S636 coamplification was performed using Hot-start protocol of QIAGEN multiplex PCR kit. The PCR conditions are summarized in **Figure 1a**. The PCR products where verified on 7.5% acrylamide gel at 22, 25, and 28 cycles to be sure of reproducibility of results and care was taken to be in the log phase of DNA amplification. Patient/normal control ratios were calculated by TotalLab TL100 software. The MLPA technique with P023 probe set was performed according to manufacturer's recommendations.

Patient/control ratios in normalized data identified 4 patients with apparently diminished marker/control gene or marker ratio. Patients P-19, P-34, P-40, and P-125 had ratios compatible with microdeletion in their corresponding region (**Figures 1b & 1c**). Other patients had ratios between 0.6 and 1.6. No increase in patient/control ratio was detected. The FISH with TUPLE1 probe had identified P-40 as the only 22q11 deleted sample, whilst the other 9 showed normal cytogenetic results. Each assay was performed in 3 independent experiments. The results of cycles 22, 25, and 28 were concordant in all patients and showed reproducibility

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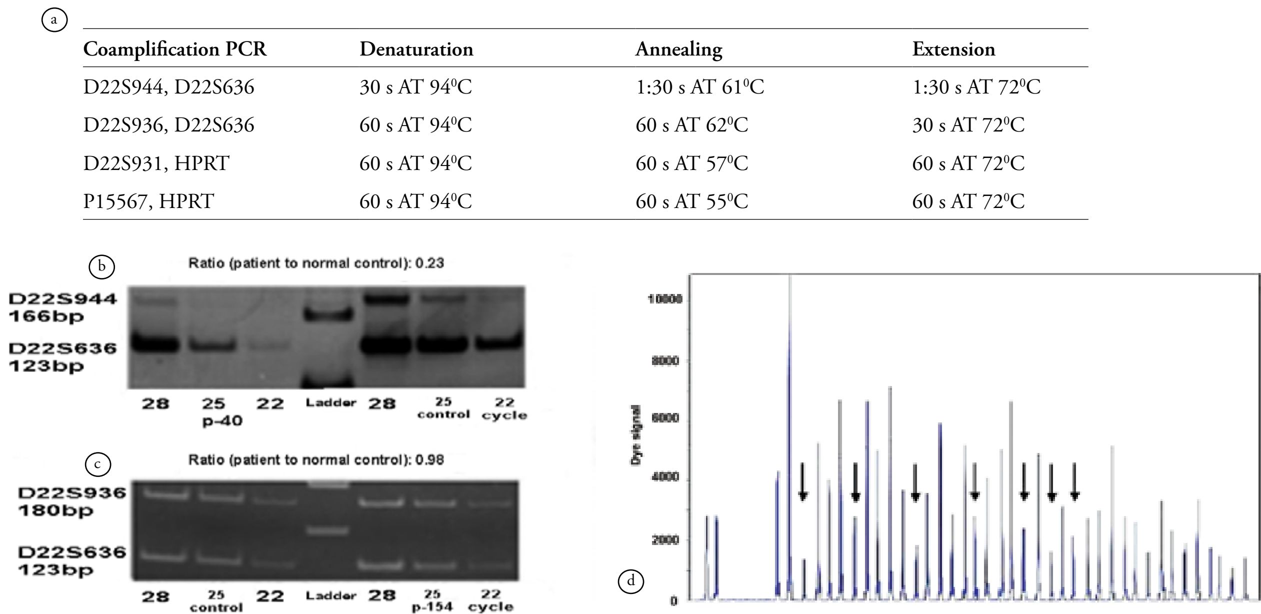

Coamplification PCR	Denaturation	Annealing	Extension
D22S944, D22S636	30 s AT 94ºC	1:30 s AT 61ºC	1:30 s AT 72ºC
D22S936, D22S636	60 s AT 94ºC	60 s AT 62ºC	30 s AT 72ºC
D22S931, HPRT	60 s AT 94ºC	60 s AT 57ºC	60 s AT 72ºC
P15567, HPRT	60 s AT 94ºC	60 s AT 55ºC	60 s AT 72ºC

Figure 1 - Semi-quantitative polymerase chain reaction (PCR) analysis of the genetic markers **a)** D22S944 **b)** and D22S936 were used against control marker D22S636. The result showed a patient to control intensity ratio of 0.23 and 0.98. **c)** Reaction conditions used in SQPCR. **d)** Multiplex Ligation-dependent Probe Amplification (MLPA) electropherograms of patient P-125 with clinical diagnosis of 22q11DS. This patient shows a microdeletion extending HIRA, CLDN5, KIAAI652, KLHL22, PCQAP, IL17R and region probes in MLPA P023 probe set.

of our experiment. The MLPA with probe set P023 confirmed results of SQPCR (**Figure 1d**). The currently accepted clinical laboratory assay for detection of microdeletions in these patients is FISH analysis with TUPLE1 or similar probes. However, many patients with phenotypic features of 22q11DS have no deletion detectable by FISH analysis. Most of these patients have atypical deletions or other chromosomal anomalies with overlapping features. We set up a new semiquantitative multiplex PCR technique to overcome drawbacks of FISH. We found 4 patients with normalized ratio below 0.6, which is considered as 'deleted'. As a control, we performed MLPA technique with probe set 023, which gather several probes in the 22q11 TDR region as well as other chromosomic regions, which have been reported to be implicated in phenotypes resembling 22q11DS. The MLPA with probe set 023 confirmed our results and delineated in more detail the deleted regions. In near 5% of patients with microdeletion, the deleted region is atypical and cannot be detected by FISH and commercial probes.[5] Our results certainly show that the molecular technique developed in this research can detect microdeletions, which are atypical and non detectable by FISH with the probe TUPLE1. In our patients, we were unable to find any microduplication. This can be explained by small sample size. Cases with features of 22q11DS and microduplications in 22q11 seem to be rare. However, molecular technique has shown to be able to find microduplications with the same sensitivity as microdeletions.

Our results confirm the presence of atypical microdeletions in Iranian patients with congenital conotruncal anomalies. This would prompt further research to implement molecular techniques in clinical practice as FISH seems not to be cost-effective and sensitive enough to detect these atypical microdeletions in Iran.

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Pethidine-induced seizure after vestibular anus repair

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Pethidine is a short acting opioid analgesic that can cause convulsions and respiratory distress in children. We are reporting a 3-month-old girl with vestibular anus who developed generalized convulsions after administration of pethidine for postoperative pain control. With multiple reports of seizures complicating pethidine therapy, it is imperative that clinicians heighten their awareness of this serious complication and reconsider using alternative analgesia for pain management.

A 3-month-old baby girl, who was apparently well, was admitted electively to the surgical ward for repair of imperforated (vestibular) anus. The patient was a full-term product of an uncomplicated pregnancy. Birth weight was 2.8 kg. Her medical history and family history were unremarkable. Development is normal for her age and there were no history of seizures. On admission to the surgical ward, the patient had normal vital signs for her age. Growth parameters were appropriate for her age and gender. Physical examination revealed a normal appearing infant apart from vestibular anus. She was neurologically normal with no neurocutaneous stigmata. Other systemic examinations were normal. Surgical repair was carried out, and the patient was placed on pethidine postoperatively for pain control with 0.5 mg/kg intravenous infusion over 15 minutes every 8 hours. Twenty minutes after receiving the third dose of the pethidine infusion, the patient developed generalized tonic convulsions with up rolling of the eyes and dusky color. Although the patient continued to breathe, she was given oxygen by facemask and intravenous diazepam 0.3 mg/kg, which aborted the seizure. Serum electrolytes (calcium, ionized calcium, magnesium, phosphate alkaline phosphatase, sodium, and potassium), blood sugar and acid base status were all normal before and after the event. There was no further episode of convulsions or adverse sequelae. Based on the previous scenario in absence of other causes of convulsion, we considered that the patient had a convulsion as a side effect of pethidine.

Pethidine (more commonly known as Meperidine or by its brand name Demerol in the United States of America) is an opioid analgesic that causes prolonged activation of opioid receptors (μ-receptors). This produces analgesia, respiratory depression, euphoria, and sedation. Pethidine is metabolized in the liver and demethylated to norpethidine. This active metabolite of pethidine has half the analgesic activity of pethidine but a longer elimination half-life (8-12 hours). At high doses of pethidine, norpethidine can accumulate; this has excitatory actions (dilated pupil, convulsions).[1] Norpethidine is toxic and has convulsant and hallucinogenic effects. The toxic effects mediated by the metabolites cannot be countered with opioid receptor antagonists such as naloxone or naltrexone and are probably primarily due to norpethidine's anticholinergic activity. The neurotoxicity of pethidine's metabolites is a unique feature of pethidine compared to other opioids. Pethidine's metabolites are further conjugated with glucuronic acid and excreted into the urine.[2] Pethidine is well known to cause seizures especially in cases where patient-controlled analgesia pumps (PCAP) are being used frequently to relieve the pain.[3,4] Pethidine neurotoxicity manifests as shakiness, tremors, myoclonus, and seizures. It is generally seen with repeated parenteral use.[5] Pethidine administered for patient-controlled analgesia within the recommended range caused seizures in some reported cases. A case was reported following oral route administration. Pethidine-associated seizures occurred in healthy children receiving pethidine for postoperative pain control as well as patients with other etiologies.[5] Seizures associated with pethidine/norpethidine toxicity can occur early during pethidine usage, and there is considerable variation in measured norpethidine levels.[6] In cases of Meperidine neurotoxicity, naloxone should not be used. Naloxone does not reverse the central nervous system (CNS) stimulatory effects of nor-Meperidine, and may actually precipitate seizure activity as the sedative effects of Meperidine are reversed, allowing the full effect of nor-

Meperidine to act on the CNS, in fact, it may aggravate the neurotoxicity. There is notably individual variability in the responses of babies to pethidine, and these patients should be observed carefully.[7] In the USA and Australia, most of the emergency units are moving away from pethidine usage in pain management considering its neurotoxicity. It is also relatively contraindicated when there is renal disease, hepatic impairment, or seizure disorders as well as hypersensitivity to Meperidine, patients who are receiving Monoamine oxidase (MAO) inhibitors or those who have received MAO inhibitors in the past 14 days (selegiline, Carbex, Eldepryl, and others). Our patient developed convulsions following pethidine administration while no other risk factors for CNS toxicity, renal or hepatic impairment was identified and there were no further episodes of convulsion after discontinuation of pethidine. These convulsions occurred with appropriate doses. Pethidine-induced seizures have been reported in multiple cases of different ages and with different etiologies, with or without patient-control devices, through either oral or parental routes. Therefore, it is imperative that clinicians heighten their awareness of these serious complications related to pethidine usage and reconsider the choices of optimal analgesia. Alternate synthetic derivatives such as fentanyl may be considered for analgesia.

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The effects of drotrecogin alpha and meropenem treatment on the levels of cytokines in rats with sepsis

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Sepsis is a systemic inflammatory reaction that is mediated by endogenous mediators that can affect a variety of organs, organ systems, and tissues. Infections caused by distinct pathogens (gram-positive or gram-negative bacteria, parasites) can present with various inflammatory manifestations including leucocytosis, leucopenia, fever or hypothermia, tachycardia, tachypnea, or other respiratory distress symptoms that may suggest the beginnings of sepsis.[1] Both pro- and anti-inflammatory cytokines and chemokines are associated with the development of sepsis as a function of their capacity to promote vasodilation, which results in vascular collapse, hypoperfusion, hypoxia, and multi-organ failure.[2] Tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), and interleukin-1β (IL-1β) are important proinflammatory mediators of sepsis. The TNF-α and IL-1β are predominantly produced by macrophages, and IL-6 is predominantly produced by hepatocytes and leukocytes. Anti biotherapy has been demonstrated to be the most effective treatment strategy regardless of the stage of sepsis. The pivotal phase 3 placebo-controlled Protein C Worldwide Evaluation in Severe Sepsis (PROWESS)[3] has identified a potential role for activated protein C (also known as activated drotrecogin alpha [DrotAA]) in the management of sepsis since it has been shown that DrotAA can confer anti-inflammatory, antithrombotic, and fibrinolytic effects. The aim of the present study was to examine the effects of DrotAA and meropenem therapies on the TNF-α, IL-1β, IL-6 serum levels in rat model of disease.

This study was approved by the Animal Care and Use Committee at Ataturk University, Faculty of Medicine Medical Experiment Application and Research Center (ATADEM), Erzurum, Turkey between 2005 and 2006, following permission from the animal research committee. Thirty Sprague-Dawley rats (200-280 g at the start of the experiment) were used for this study and were allowed free access to food and water. Animals were fasted for 8-10 hours prior to the start of the experimental procedures at which time were anesthetized, had blood drawn and their external jugular vein catheterized. Septic peritonitis was induced using the cecal ligation and puncture (CLP) method of Wichterman et al.[4] Five experimental groups were examined: 1. Control group (group I), untreated, catheterized only; 2. Sham-

treated control (group II), treated as group I but were also subjected to laparotomy but not CLP; 3. Sepsis group (group III), CLP positive control group; 4. Sepsis+DrotAA group (group IV), CLP-treated rats also treated with a DrotAA (Xigris®, Lilly, USA) infusion, 100 µg/kg administered over a 30 minute period 48 hours after CLP induction; 5. Sepsis + DrotAA + meropenem group (group V), CLP-treated rats also treated with DrotAA and Meropenem (Meronem®, Astra Zeneca, Switzerland) administered at 30 mg/kg/day intravenously for 3 days. At 3 and 48 hours post DrotAA treatment, groups 4 and 5 were sedated using ketamine and a sternotomy was performed. Five ml of blood was taken from the right ventricle and all animals survived without complications throughout the observation period (from CLP to collecting blood samples). Blood samples were subsequently centrifuged at 4000 rpm for 10 minutes to obtain serum that was stored at -85°C until the levels of TNF-α, interlukin (IL)-1β, and IL-6 were quantified using either a rat TNF-α enzyme linked immunosorbent assay (ELISA) kit (Rat TNF-α Immunoassay Kit, Invitrogen, Carlsbad, CA), rat IL-1β ELISA kit (Rat IL-1β Immunoassay Kit, Invitrogen) or a rat IL-6 ELISA kit (Rat IL-6 Immunoassay Kit, Invitrogen) (unit values pg/ml). Cytokine levels for each of the 5 groups were calculated and the data expressed as the mean±standard error (SE). Statistical analyses were carried out using the Mann-Whitney U test and differences were considered to be significant if $p<0.05$.

The following profiles were obtained for the respective cytokines examined: TNF-α; the only significant differences in TNF-α levels were detected between the sepsis group (group III) and groups I and II ($p<0.05$); IL-6, there were no statistical differences in the IL-6 levels between groups I and II or groups III and IV. However, the IL-6 levels in group III were significantly elevated ($p>0.01$) compared to group II (sepsis versus sham-treated groups) and IL-6 levels were significantly reduced in groups IV and V when compared to group III ($p<0.05$); IL-1ß, there were no significant differences in the IL-1ß serum levels between groups I and II or between groups IV and V. However, serum IL-1β levels were significantly greater in the septic rats (group III) in comparison to group II ($p<0.05$), and these levels were significantly reduced following treatment (groups IV and V) ($p<0.05$).

Inflammatory mediators are important mediators of inflammation and immune function and can be generated in response to different infectious agents. Their production is linked to sepsis and other related conditions, for example, SIRS (systemic inflammatory response syndrome). Patients being treated for sepsis and sepsis-related conditions present with elevated levels of both anti-inflammatory and proinflammatory cytokines such as TNF-α, IL-1β, and IL-6.[5,6] Patients that do not survive episodes of SIRS, sepsis, or severe sepsis presented with elevated levels of IL-1β, and IL-6 compared to patients that survived similar episodes.[7] This parallels our study that showed that treatment-related decreases in IL-1β and IL-6 following treatment correlated with survival. Treatment of sepsis in this study did not reduce the TNF-α levels that were elevated in the sepsis group. This observation supported previous reports that also demonstrated that elevated levels of this cytokine were not associated with mortality.[7] Changes in the TNF-α levels in the treatment groups had no effects on the experimental outcome. The effects of DrotAA-therapy on the mortality of patients with severe sepsis was shown in the PROWESS study that was randomized, double blind and placebo-controlled.[3] There were no significant differences in TNF-α levels between DrotAA- and/or meropenem-treated (100 µg/kg and 30 mg/kg/d each) or untreated rats in our study. The data presented in this report demonstrated that the IL-6 levels significantly decreased following DrotAA therapy. The question remains whether IL-6, which has a short half-life can be used as an accurate marker indicating successful sepsis treatment even though the PROWESS study indicated that reduced IL-6 levels correlated with recovery from a septic episode.[8] In addition, IL-6 has been reported to be an important marker in the prognosis of anti-inflammatory and anti-thrombotic therapy along with APACHE II scores.[1] Although IL-1β can facilitate the regulation of proinflammatory processes and its levels increase during sepsis, its role in the progression and resolution of sepsis are not well understood. When IL-1β levels were evaluated in terms of treatment success, serum levels were not significantly different between survivors and non-survivors.[7] When IL-1β was evaluated in terms of treatment it did not correlate as well with success as did the IL-6 response. In our CLP sepsis model, TNF-α, IL-1β, and IL-6 levels were elevated, but TNF-α was not significantly reduced by the combination of DrotAA or DrotAA+ meropenem, suggesting that measuring TNF-α levels was not useful in predicting the success of respective sepsis therapies. In contrast, IL-6 levels correlated with disease presentation and monitoring the levels of this cytokine may be a useful strategy in the management of sepsis.

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