

Differential Serum Proteomes between Subphenotypes of Nonsyndromic Cleft Lip with or without Cleft Palate

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Abstract

Nonsyndromic cleft lip with or without cleft palate (nsCL/P) accounts for the majority of orofacial clefting in humans. New insights into the etiology of nsCL/P are requiring diversified and complementary investigative technologies. Since proteins act as the functional end products of gene expression, this investigation examined some distinct protein expression profiles of genes related to nsCL/P by analyzing differentially optimized peripheral serum protein mass spectrum peak values, utilizing Enhanced Laser Desorption Ionization Time of Flight (SELDI-TOF) technology. The differential peak values of protein 4119.1711 and 5639.1775 were discriminated respectively between cleft children and normal controls. 4079.4151, 5482.6131, 5684.2834, and 33310.9487 were cleft boys versus cleft girls. 2745.7079, 4473.4883, 6454.9339, 6560.7769, and 6654.3226 were cleft lip only versus cleft palate only. 4178.7872, 7842.6306, and 8147.0166 were cleft lip only versus cleft lip and palate. 4160.0878 and 5708.5676 were cleft lip only versus normal controls. These results indicate to be assistant for genomic etiology of nsCL/P.

Keywords: Proteomics; Genetics; SELDI-TOF-MS; Congenital abnormality; Nonsyndromic cleft lip with or without cleft palate (nsCL/P)

INTRODUCTION

Nonsyndromic cleft lip with or without cleft palate (nsCL/P) is one of the most common congenital deformities, with an incidence of 0.5-1.0% world widely and a male to female ratio as 1.5-2.0 : 1.0. In contrast to the less common syndromic forms of CL/P, which are often due to single genes that cause maxillofacial defects accompanied with other systemic diseases or malformations, the major 50-70% of clefts are nonsyndromic which are generally acknowledged to be caused by multiple susceptibility genes as well as environmental factor¹⁻⁶. Correlations have been found between MSX1, MYH9, WNT, PVRL3, TGF α , RAR α , BMP4, TGF β ₂, TGF β ₃, MSX, BCL₃, F13 α or IRF6 and nsCL/P by scholars from gene territory^{2,5,7-16}. However, results can differ, due to the complexity of the etiology, the uncertainty of the inheritance

patterns, the limitations of Genotyping studies, and differences among study samples¹⁷⁻¹⁹. With a new insight, the application of proteomics may increase our understanding of nsCL/P, since gene mutations are manifested via proteins, and hence there should be certain differentially expressed proteins that may be discovered. We applied the proteomic technique of Surface Enhanced Laser Desorption Ionization Time of Flight (SELDI-TOF) to cleft patients and normal human controls in this research, expecting to learn more etiology about nsCL/P.

METHODS

Clinical Data

Diagnoses were confirmed by excluding the syndromes known to us, including Van der Woude syndrome, Kabuki syndrome, Meckel syndrome. After consent

was granted and prior to any therapeutic intervention, fasting peripheral blood samples were obtained in the early morning from pediatric patients in the Oral and Maxillofacial Surgery Department of Second Affiliated Hospital, School of Medicine, Zhejiang University from March 2005 to January 2006. The experiments and control subjects were both comprised of 18 boys and 12 girls aged 0.3-6 years, respective 30 subjects for experimental and control groups. Control groups were from healthy children outpatients for regular dental examinations. Sera were separated at once and stored at -20°C until use. The experimental group was divided into cleft lip only, cleft palate only, cleft lip and palate. No biological kinship existed among the subjects.

Laboratory Procedures

To use SELDI-TOF biotechnology we included serum, urine, secretions, cell lysate, and tissue sample proteins with energy absorbing molecules on the chip after removing impurities and other contaminants with buffer and drying. Following thawing and 5 minutes of centrifugation (3 000 r/min), 10 μl sample was added to 90 μl 0.5% U9 (9 mol/L urea, 0.15% CHAPS (3[[3-cholamidopropyl] dimethylammonio]-1-propanesulfonate) pH7.14, and incubated for 60 min at 4°C in albumin affinity resin (CibacronBlue 3GA, Sigma) with 600 r/min vigorous shaking. After 5 minutes centrifugation at 2000 r/min, 50 μl supernatant was diluted to 200 μl with 20mmol/L HEPES pH7.14, and was installed into a 96-well Bioprocessor (Ciphergen). Samples were incubated for 60 min at 4°C with 600 r/min vigorous shaking, and the serum discarded. The chip was washed 3 times for 5 min each with 200 μl of 20mmol/L HEPES pH7.14, and 2 times for 1 min each with deionized water, 0.15 μl energy absorbing molecule CHCA (Ciphergen) was added after drying, and duplicated one time.

Seldi-TOF Biotechnology

Samples were desorpted and ionized by laser and then accelerated to fly out through a vacuum tube and captured by the ion detecting receiver. Ciphergen SELDI Protein Biology System II plus (PBS II plus) and ProteinChip Software (Version 3.0, Ciphergen Biosystems) were used to read the chips and analyze

the data. The square of the ion flight time is in inverse proportion to the ratio of molecule size and molecular charge. When the numbers of usually single charge in the tube^{20,21} are equal, the time of flight differs with the molecule size. The smaller the molecule is, the shorter the flight time would be; hence small molecules are first to reach the receiver. As reflected in a mass spectrum diagram, the peak position corresponds to molecular weight of the relevant protein compositions, and the spectral peak height corresponds to the number of the relevant protein compositions. Therefore, proteins can be identified by peak position and height. Differential proteins can be discriminated by comparison to the high-throughput spectrums between groups.

Laser intensity 135, 65 laser shots per sample, detector sensitivity 7, and automatically-detected peaks from 2000 to 30 000 m/z were used. Mass accuracy was calibrated to less than 0.1% using the All-in-1 peptide molecular mass standard (Ciphergen Biosystems). The peaks were normalized and simultaneously existed in more than 10% of samples with a bias lower than 0.3% for the same protein m/z peak in different samples. Noises were filtrated (first signal to noise ratio > 2.2 , second valued as 1). Multivariate analysis of variance Kruskal-Wallis was used to compare the data of different groups and discrepant mass peaks groups were found and established.

RESULTS

The statistically significant protein peak values of mass to charge ratio are illustrated in Table 1. Correlation comparisons between the differential protein peaks and the pathway proteins for such cell factors as $\text{TGF}\alpha$, $\text{TGF}\beta_2$, $\text{TGF}\beta_3$, MSX , BCL_3 , $\text{F13}\alpha$, MYH9 , WNT , PVRL3 , $\text{TGF}\alpha$, $\text{RAR}\alpha$, BMP4 , and IRF6 were conducted through <http://us.expasy.org/>. The differential peak values of protein 4119.1711 and 5639.1775 were discriminated respectively between cleft children and normal controls (table 1). 4079.4151, 5482.6131, 5684.2834, and 33310.9487 were cleft boys versus cleft girls. 2745.7079, 4473.4883, 6454.9339, 6560.7769, and 6654.3226 were cleft lip only versus cleft palate only. 4178.7872, 7842.6306, and 8147.0166 were cleft lip only versus cleft lip and palate. 4160.0878 and 5708.5676 were cleft lip only versus normal controls

Table 1. Differential protein peak values.

Group	Differential protein peak values (p<0.01)
Cleft children versus Normal controls	4119.1711, 5639.1775
Cleft boys versus Cleft girls	4079.4151, 5482.6131, 5684.2834, 33309.9487
Cleft lip only versus Cleft palate only	2745.7079, 4473.4883, 6454.9339, 6560.7769, 6654.3226
Cleft lip only versus Cleft lip and palate	4178.7872, 7842.6306, 8147.0166
Cleft lip only versus Normal controls	4160.0878, 5708.5676

DISCUSSION

To date, similar with SCL/P, studies for nsCL/P also concentrated on single gene etiology. In fact, multiple genes defect and other factors account for nsCL/P. Although researches have demonstrated that any deficit or variation of one single gene involving embryonic fusion such as Epidermis Growth Factor (EGF) or MYH9, WNT, PVRL3, TGF α , RAR α BMP4, could exacerbate initiation events and lead to the occurrence of a cleft, it is indispensable to study some feature proteins characteristically correlated to the formation of clefts. If they diminish the clinical expression of cleft or revise it to normal via reconstruction of the executive function of key proteins, guidance for treatment and intervention for the formations of cleft lip and palate would be achieved, considering that the morphological defect could be due to mutual effects among multiple cells under the control of numerous structural and regulatory proteins, as well as mutual action between cytological differentiation and migration of neural crest cells.

Furthermore, due to the high throughput of data and the comparison of tremendous protein molecules and multipeptide sequences, to understand the ontogeny and embryology of the disease from gene to protein and cell function and through to clinical manifestation, is far beyond the single capability of 2-dimensional electrophoresis, while, Surface Enhanced Laser Desorption Ionization Time of Flight (SELDI-TOF), on the other hand, affords greater technical capacity for functional proteomic research.

With regard to the etiology of nsCL/P, although genetic reports have inferred several candidate genes^{2,7-14}, there are seldom insights into the functionally dynamic steps in the pathology—from gene to protein to cell function and through to clinical manifestation—how transcription and translation are altered and how the altered proteins execute their functions. Since pathological study has suggested that the appearance of nsCL/P is evoked through a series of mutually affected

protein receptors, it has become a novel starting point to research the functionally executive proteins for disease. Within this investigation, diversification of hormones and protein volume related to adolescence was avoided by limiting subject ages to less than 6 years old, as was also done on the familial patients to exclude the intervention of any familial inherited genes. The appearance of differential protein peak values 4119 and 5639, which were discriminated between cleft pediatric patients and normal controls (table 1), respectively, infers the existence of differential functional proteins. Even though they could not be judged to be genetically correlated receptor proteins, this research still, reveals the existence of differential proteins or multipeptides between cleft and normal subjects.

Secondly, this study provides additional verification that the mechanisms of cleft lip with or without palate (nsCLP) are different from single cleft palate (CP), and single cleft lip with single cleft palate. The bigger quantity of differential proteins as 3-5 differentially distinguished peaks in respective cleft groups (table 1) suggests that the protein pathways for mechanisms of pure cleft lip or palate or cleft lip with palate are discrepant. This concurs with many scholars' views^{1,17,18}, based on embryologic and epidemiologic evidence, that the primary and secondary palate are separately formed. Furthermore, the simultaneous existence of nsCL/P and purely cleft palate in the same family is very rarely found, supporting the distinction between affected genes. In contrast, some current quantitative genetics researchers argue that a few factors leading to nsCL/P are also pathological for CP²².

Thirdly, the establishment of clinical phenotypes according to the severity of the cleft is confirmed. From the results of this research, the observation of differential proteins illustrates that, even for pathologically visible clefts, there exist different degrees of protein/receptor regulation that impact the severity and phenotype of the cleft. The differential

proteins, evidently structural and regulatory proteins, comprise the pathways leading to the fusion of facial or palate protrusions and signal cellular differentiation and interaction as well as neural crest migration, eventually and directly bringing about the clinical manifestations.

Within the limitations of this current work, we include small sample size and the distance to genetics, genealogy, and epidemiology. When comparing the mass/charge of the differential proteins with the database at <http://us.expasy.org/>, the dispersion of the differential proteins was found to be limited, concentrated on certain PI values, which have not yet been reported to have any relationship with the incidence of cleft lip or palate, despite the exclusion of hormone and familial proteins. Larger samples such as enrollment of adolescents and familial subjects should strengthen the results. The research results should not be inferred to be the actual source of dysmorphism¹⁵. This research also depends on the temporary limitations of the genes included for study and Subsequent study would include genes such as MTHFR and BCL3, which may have weaker effects on clefting.

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