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基于 iTRAQ 定量蛋白质组学方法筛选鼻咽癌放射抗拒相关蛋白

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摘要 **目的** 比较不同放射敏感性鼻咽癌患者血清蛋白质表达差异,筛选出与鼻咽癌放射抗拒性相关的蛋白质。**方法** 提取不同放射敏感性鼻咽癌患者血清蛋白,应用核素标记相对和绝对定量(iTRAQ)技术标记蛋白,联合液相色谱和串联质谱(LC-MS/MS)分离、分析肽段。采用 Proteome Discoverer 1.4 软件对蛋白进行鉴定和定量分析,对获得的差异蛋白进行 GO 分析。**结果** 共鉴定出差异表达的蛋白质 65 个,其中上调 34 个、下调 31 个,GO 分析显示差异表达的蛋白质主要涉及生物学过程调控、压力应激反应及分解代谢等生物学过程。**结论** S100A7、胰岛素样生长因子结合蛋白 2、α1 抗胰蛋白酶和热休克蛋白等差异表达的蛋白质可能与鼻咽癌放射抗拒性相关。

关键词 鼻咽癌;辐射抗拒;蛋白质组学

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鼻咽癌是我国常见恶性肿瘤之一,其主要治疗方法为放射治疗,辅助以化疗及手术等综合治疗。早期是以单纯放射治疗为主,而中晚期是以放化综合治疗为主要治疗手段。尽管放射治疗已取得较好的疗效,然而放射抗拒所致的局部失败和远处转移仍较常见[1]。肿瘤细胞放射敏感性差异是导致局部复发率及残留率高的一个重要原因。研究[2]表明当人体主要携氧工具 - 血红蛋白浓度下降时,血氧含量减少,鼻咽癌患者表现为放射抗拒,局部控制率明显降低。准确地评估肿瘤放射敏感性是实行个体化方案治疗至关重要的一步,也是临床亟待解决的问题。该研究采用同位素相对标记与绝对定量标记技术(isobaric tags for relative and absolute quantitation,iTRAQ)与二维液相色谱(liquid chromatography-tandem mass spectrometry,LC-MS/MS)联用质谱分离鉴定技术,分析寻找与放射抗拒相关的蛋白,从而为个体化治疗奠定基础。

growth factor (bFGF) in promoting bone formation. ***Methods*** 28 cases with periodontitis, whose loose teeth couldn't be retained and then were removed, were randomly divided into three groups. The PRF group was filled with the mixure of deproteinized bovine bone mineral (Bio-Oss) and PRF in the alveolar fossa. The bFGF group was used bFGF and Bio-Oss, and the control group was only added Bio-Oss. The clinical measurements of changes in vertical bone resorption in the surgical area, average value of bone density, bone height of adjacent teeth were done after six months of operation. The bone specimens were taken out from PRF and bFGF groups to be observed. And the professional image analysis software IPP6.0 was used to conduct quantitative analysis of the new bone quantity. ***Results*** The control group was respectively compared with PRF and bFGF group in the amount of vertical bone resorption, the average bone density, the variation of adjacent alveolar bone height and the percentage of new bone, and they were statistically significant ($P < 0.05$); while the PRF group was compared with bFGF group, they were statistically significant in the average bone density ($P < 0.05$) and the percentage of new bone ($P < 0.05$). However, there was no statistical significance in the amount of vertical bone resorption and the variation of adjacent alveolar bone height. Therefore, the ability of PRF group to promote bone formation was higher than that of bFGF group. ***Conclusion*** PRF can more effectively promote the formation of bone tissue, which can obtain more new bone, and provide a good environment for implant restoration.

Key words PRF; bFGF; periodontitis; site preservation; histologic

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1 材料与方法

1.1 病例资料 收集2013年8月~2015年7月于安徽医科大学附属省立医院病理确诊的鼻咽癌患者血清样本40例。清晨6点空腹取静脉血5 ml于促凝管内,室温放置2 h,4 ℃恒温离心机2 500 r/min离心20 min,置于-80 ℃冰箱分装保存。鼻咽部照射40 Gy时MRI检查结果作为放射敏感性的评价指标,根据WHO实体瘤客观疗效评价标准,肿瘤消退<50%定义为放射抗拒,>50%定义为放射敏感,分为放射敏感组与放射抵抗组[3]。每组选取标本20例,两组患者性别、年龄、分期等临床病理因素差异无统计学意义。

1.2 去除高丰度蛋白 各组的20例样本以等体积混合,采用去血清高丰度亲和色谱柱Agilent Multiple Affinity Removal LC Column-Human 14去除高丰度蛋白质,得到低丰度组分溶液。应用10 ku超滤管进行超滤浓缩,加入一倍体积的SDT裂解液,沸水浴15 min,13 400 r/min离心20 min,取上清液。采用BCA法进行蛋白质定量。分装样品,-80 ℃保存。

1.3 SDS-PAGE电泳 各样品取蛋白质20 μg分别加入5×上样缓冲液,沸水浴5 min,进行12.5% SDS-PAGE电泳(恒流14 mA、90 min),考马斯亮蓝染色。

1.4 FASP酶解 各样品取30 μl蛋白质溶液,分别加入DTT至终浓度为100 mmol/L,沸水浴5 min,冷却至室温。加入200 μl UA buffer混匀,转入10 ku超滤离心管中,13 400 r/min离心15 min,弃滤液;加入100 μl IAA buffer(100 mmol/L IAA in UA),600 r/min振荡1 min,室温避光反应30 min,13 400 r/min离心15 min;加入100 μl UA buffer,13 400 r/min离心15 min;加入100 μl 10倍稀释的Dissolution buffer,13 400 r/min离心15 min;加入40 μl Trypsin buffer(4 μg Trypsin in 40 μl Dissolution buffer),600 r/min振荡1 min,37 ℃放置16~18 h;换新收集管,13 400 r/min离心15 min;再加入40 μl 10倍稀释的Dissolution buffer,13 400 r/min离心15 min,收集滤液。采用C_{18} Cartridge对肽段进行脱盐,肽段冻干后加入40 μl Dissolution buffer复溶,肽段定量(OD_{280})。

1.5 iTRAQ试剂标记 两组样本各取100 μg用于iTRAQ试剂标记,放射敏感组和抗拒组分别标记以113和116试剂,具体步骤参照美国AB公司iTRAQ试剂盒说明书进行,进行3次技术重复。

1.6 强阳离子交换色谱(strong cation exchange,SCX)分级 将每组标记后的肽段混合,采用AKTA Purifier 100进行分级。缓冲液A液为10 mmol/L KH_2PO_4,25% ACN,pH 3.0;B液为10 mmol/L KH_2PO_4,500 mmol/L KCl,25% ACN,pH 3.0。色谱柱以A液平衡,样品由进样器上样到色谱柱进行分离,流速为1 ml/min。

1.7 质谱鉴定 每份样品采用纳升流速的HPLC液相系统Easy nLC进行分离。缓冲液A液为0.1%甲酸水溶液,B液为0.1%甲酸乙腈水溶液(乙腈为84%)。样品经色谱分离后用Q-Exactive质谱仪进行质谱分析。检测方式为正离子,母离子扫描范围300~1 800 m/z。

1.8 统计学处理 采用SPSS 13.0软件进行分析。质谱数据采用Mascot 2.2和Proteome Discoverer 1.4进行数据库搜索,数据库为uniprot_human。符合表达差异倍数大于1.2倍(上下调)且$P<0.05$筛选标准的蛋白质视为差异表达蛋白质。鉴定得到的差异蛋白通过生物信息学方法使用GO注释及富集分析,分别从生物学进程(biological process,BP)、细胞成分(cellular component,CC)和分子功能(molecular function,MF)这3方面进行基因显著性分析。通过Fisher精确检验来评价某个GO term蛋白质富集度的显著性水平。

2 结果

2.1 差异表达蛋白质的筛选 质谱共得到3 780个唯一多肽片段,经数据库分析后,共获得457个蛋白,其中有65个差异表达的蛋白质,其中,放射抗拒组相对于放射敏感组上调差异表达蛋白质34个(表1),下调差异表达蛋白质31个(表2)。

2.2 差异表达蛋白质GO基因功能分类 对筛选的65个差异表达蛋白质进行GO功能注释,在生物学进程分析结果中,差异表达蛋白质主要参与包括生物学过程调控、压力应激反应、分解代谢等主要进程。其中12%的蛋白质参与生物学过程调控,S100A7参与生物学过程调控;压力应激反应的蛋白质占12.0%,补体因子H相关蛋白、热休克蛋白(heat shock protein,HSP)和胰岛素样生长因子结合蛋白-2(insulin-like growth factor binding protein-2,IGFBP2)都参与了压力应激反应;与分解代谢有关的蛋白质占11%,α1抗胰蛋白酶处于这一进程中;其他的蛋白质与信号传导、生物学的黏附和免疫应

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表 1 上调部分差异表达的蛋白质

登录号	蛋白质名称	覆盖率(%)	等电点	比值
P20851	C4b-binding protein beta chain	21.43	5.14	1.34
P78492	Inter-alpha-trypsin inhibitor	74.51	4.89	1.38
P19652	Alpha-1-acid glycoprotein 2	35.82	5.11	1.29
Q03591	Complement factor H-related protein 1	51.82	7.39	1.31
P00738	Haptoglobin	37.44	6.58	1.52
H7C0N0	Inter-alpha-trypsin inhibitor heavy chain H1	14.66	6.19	1.30
H0YFH1	Alpha-2-macroglobulin	24.66	10.01	1.51
P08519	Apolipoprotein(a)	28.19	5.88	1.34
K7ERI9	Apolipoprotein C-I	23.38	6.71	1.29
Q04756	Hepatocyte growth factor activator	24.43	7.24	1.23
Q15485	Ficolin-2	11.82	6.77	1.21
P02763	Alpha-1-acid glycoprotein 1	36.82	5.02	1.41
P05090	Apolipoprotein D	13.23	5.15	1.26
P01876	Ig alpha-1 chain C region	19.83	6.51	1.23
A0A075B6N8	Ig gamma-3 chain C region	34.22	7.90	1.38
A6XGM4	BORIS transcription factor transcript variant C5	2.11	4.82	1.45
Q02985	Complement factor H-related protein 3	18.79	7.55	1.44
P18065	Insulin-like growth factor-binding protein 2	15.69	7.50	1.21
P04275	von Willebrand factor	23.89	5.48	1.44
Q6LAM1	Heavy chain of factor I	50.16	7.59	1.20
B4E1C2	Kininogen 1, isoform CRA_b	43.94	6.81	1.24
P02042	Hemoglobin subunit delta	44.90	8.05	1.26
P02652	Apolipoprotein A-Ⅱ	59.00	6.62	1.27
G3V2B9	Alpha-1-antitrypsin	39.05	4.64	1.27
P05109	Protein S100-A8	33.33	7.03	1.21
B4DXY3	Heat shock 70 ku protein	5.83	6.49	1.23
V9H1D9	Alpha globin	26.56	8.24	1.37
P07225	Vitamin K-dependent protein S	40.83	5.67	1.31
P01011	Alpha-1-antichymotrypsin	59.10	5.52	1.21
Q86SV5	Complement C2	41.94	4.65	1.47
P01023	Alpha-2-macroglobulin	40.91	6.46	1.28
K7ER74	Protein APOC4-APOC2	33.15	6.64	1.22
P61769	Beta-2-microglobulin	49.58	6.52	1.27
P11226	Mannose-binding protein C	50.00	5.49	1.22

答过程有关。在亚细胞定位分析中,分布在细胞器和胞外的蛋白质各占 24%,其他还有一些蛋白质属于细胞膜、胞外区和细胞连接的成分。在分子功能分析中,其中结合蛋白质占 51%,差异表达蛋白有催化活性、酶调节活性和转运活性。

3 讨论

鼻咽癌的放射敏感性是影响疗效的重要因素。如果在放疗前能够对个体肿瘤的放射敏感性进行预测,可有助于选择合适的个体化治疗方案,提高肿瘤控制率,减少正常组织放射损伤。目前,相关研究[4]已证实放射敏感和放射抗拒的鼻咽癌细胞株,其蛋白质表达存在差异,部分蛋白表达上调或下调。然而,其仅仅为细胞实验,并不能完全反映人体状况,更难以在临床使用。而血清标本的优势明显,有标本易于采集和所需的标本量较少等优点,便于临床使用。

高通量蛋白质组学技术为本研究肿瘤放射敏感性提供了新的手段,蛋白质的结构和功能最终影响着生命活动的变化,寻找与放射抗拒相关的蛋白用于个体化方案制定具有重要意义,不仅有较高的临床应用价值,而且对揭示放疗抗拒机制意义重大。

本研究利用 iTRAQ 技术筛选放射敏感和放射抗拒差异蛋白,放射抗拒组相对于放射敏感组上调差异表达蛋白质 34 个,下调差异表达蛋白质 31 个。初步鉴定出的有明确功能的蛋白分别涉及到几种不同的生物过程:生物学过程调控、压力应激反应、分解代谢等主要进程。

S100A7 是 S100 蛋白质家族成员,可调节细胞的增殖、凋亡、信号转导等,是头颈部肿瘤的不良预

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表 2 下调部分差异表达的蛋白质

登录号	蛋白质名称	覆盖率(%)	等电点	比值
P02787	Serotransferrin	44.41	7.12	0.62
Q7Z7Q0	APOB protein	64.85	7.42	0.62
Q8NBP7	Proproteinconvertasesubtilisin/kexin type 9	6.94	6.61	0.76
P31151	Protein S100-A7	10.89	6.77	0.75
D6W633	Protein tyrosine phosphatase, receptor type, S	0.84	6.24	0.62
A0A087WUM5	Golgi-associated plant pathogenesis-related protein 1	48.61	10.21	0.77
P08294	Extracellular superoxide dismutase [Cu-Zn]	29.17	6.61	0.80
Q86U17	Serpin A11	15.40	7.68	0.71
J3QLR4	Angiotensin-converting enzyme	12.61	4.67	0.71
P00390	Glutathione reductase, mitochondrial	5.56	8.50	0.68
Q1W658	Follicle-stimulating hormone beta subunit	14.47	6.73	0.49
Q0ZCH9	Immunglobulin heavy chain variable region	23.14	8.46	0.82
I3L1Q6	Phosphatidylcholine-sterol acyltransferase	13.08	7.24	0.67
P16930	Fumarylacetoacetase	2.86	6.95	0.62
Q59FD0	Huntingtin interacting protein-1-related	6.24	6.65	0.80
E9PP27	Reticulocalbin-1	22.41	4.53	0.83
Q9BZQ0	Follistatin-related protein	9.52	4.70	0.79
A0A0A0N0L5	IL6ST isoform 3	4.41	5.94	0.82
F5GZZ9	Scavenger receptor cysteine-rich type 1 protein M130	6.13	6.10	0.80
Q1JUQ3	FK506 binding protein12	35.14	5.78	0.81
O43532	RIG-like 7-1	13.45	6.79	0.79
Q5HY54	Filamin-A	1.99	6.05	0.83
H0YLU3	Alpha-mannosidase 2x(Fragment)	6.27	7.91	0.70
Q59FI2	Protein tyrosine phosphatase, receptor type, F isoform 2 variant	2.96	6.64	0.78
K7EM16	Vasodilator-stimulated phosphoprotein	6.56	7.94	0.69
L8E853	von Willebrand factor	24.49	5.48	0.73
Q9UK55	Protein Z-dependent protease inhibitor	41.67	8.27	0.82
Q6EMK4	Vasorin	10.10	7.39	0.74
P10720	Platelet factor 4 variant	48.08	9.10	0.80
Q13103	Secreted phosphoprotein 24	9.95	8.32	0.83
Q8TCZ8	Apolipoprotein E(Fragment)	64.91	9.98	0.73

后指标[5]。可通过晚期糖基化终末产物(receptor for advanced glycation end products,RAGE)介导的内分泌机制在肿瘤局部招募基质金属蛋白9、环氧化酶2等促炎症因子表达,促进肿瘤血管再生及肿瘤增殖[6]。研究[7]显示在乳腺癌细胞中,S100A7可促进核转录因子-κB(nuclear factor-κB,NF-κB)DNA结合活性和基质金属蛋白9和血管内皮生长因子表达,从而促进肿瘤细胞增殖和侵袭。而NF-κB参与放射治疗抗拒的相关通路,活化可导致肿瘤细胞对放射治疗的抵抗[8]。这可能是S100A7导致放疗抗拒的原因。

IGFBP2在多种肿瘤中明显升高,与肿瘤侵袭相关,影响患者预后[9-10]。Guo et al[11]发现在肺癌患者血清中IGFBP2明显升高,且与肿瘤大小分期明显相关,血清浓度升高患者生存时间缩短。Uzoh et al[12]发现IGFBP2促进前列腺癌细胞生长,而且增加化疗抗拒,抑制表达可增加化疗敏感性。

α1抗胰蛋白酶浓度在炎症、感染和肿瘤时会明显增加,研究[13]显示在肺癌和前列腺癌中均升高,随着分期增加而明显升高。

HSP是机体存应激条件下合成的应激蛋白质,参与调节细胞的增殖、分化和细胞抗凋亡的信号转导,与放射抗拒性相关。Du et al[14]发现对放射抗拒的子宫内膜癌细胞采用沉默HSP70后,细胞的凋亡率明显增加,抑制HSP70表达可明显增加放射治疗效果。王润文 等[15]发现放疗后有残留鼻咽癌患者HSP70阳性表达率明显高于病灶完全消失患者,两组相比差异有统计学意义。作者认为HSP70是影响鼻咽癌放射抗拒的重要因素。

本实验结果提示,iTRAQ技术是鼻咽癌放射敏感性研究科学可靠的预测方法,可以精确地筛选出肿瘤辐射抗拒的相关蛋白。然而,由于肿瘤的放射抗拒机制复杂,涉及到多种复杂因素相互作用,其分子机制仍不清楚。本研究尚处于初级阶段,筛选出蛋白质在放射抗拒中的具体机制仍不明确,还需要进一步研究。

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Screening of radioresistant associated proteins in nasopharyngeal carcinoma by iTRAQ quantitative proteomics

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Abstract ***Objective*** To discover the proteins related to nasopharyngeal carcinoma(NPC) radioresistance by screening differentially expressed proteins in NPC patients with different radiosensitivity. ***Methods*** Two groups of sera were respectively collected from NPC patients with different radiosensitivity(20 casesineach group). Isobaric tags for relative and absolute quantitation(iTRAQ)-taggingcombined with liquid chromatography tandem mass spectrometry(LC-MS/MS) analysis was used to identification of differentially expressed proteins between the two groups. The identification and quantitation of the proteins were analyzed by Proteome Discoverer 1.4 software. The proteins differentially expressed were analyzed by GO(Gene Ontology) terms. ***Results*** A total of 65 differentially expressed proteins were identified,of which 34 were up-regulated and 31 were down-regulated. GO analysis showed these proteins to be involved in keybiological processes such as biological regulation,response to pressure stress and catabolism. ***Conclusion*** Analysis identify the following 4 proteins that may be the candidate biomarkers for predicting radioresistance of NPC: S100A7, insulin-like growth factor-binding protein 2, alpha-1-antitrypsin and HSP 70.

Key words nasopharyngeal carcinoma; radioresistance; proteomics

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