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Six Candidate Proneural Factor Messenger RNAs for the Differentiation of Neuroendocrine and Non-Neuroendocrine Carcinomas of the Lung

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Abstract

Background: Lung neuroendocrine carcinomas, i.e., small cell lung carcinoma (SCLC) and large cell neuroendocrine carcinoma (LCNEC), and non-neuroendocrine carcinomas, e.g. squamous cell carcinoma and adenocarcinoma are both thought to arise from the same endoderm as respiratory epithelium. However, it is not clear how neuroendocrine carcinomas acquire and maintain their neuroendocrine features. To date, 19 proneural factors that function in the development of the fetal neural system and differentiation of neuroendocrine cells of endodermal origin have been identified. In this study, we investigated the specificity of proneural factor expression in SCLC, lung LCNEC, lung squamous cell carcinoma and lung adenocarcinoma.

Methods: RNA was extracted from 3 SCLCs, 3 LCNECs, 10 invasive squamous cell carcinomas and 10 invasive adenocarcinomas. Specific PCR primers were generated for the 19 proneural factors and messenger RNA copy numbers were measured using reverse transcription real time PCR. Differences in expression were then statistically analysed.

Results: Insulinoma-associated protein 1(*INSM1*) and mammalian achaete-scute homolog (*MASH*) *1* mRNA was significantly higher in SCLCs than in squamous cell carcinomas. Oligodendrocyte transcription factor (*OLIG*)*1* and mammalian atonal homolog (*MATH*)*2* mRNA levels were significantly lower in SCLCs and squamous cell carcinomas than in adenocarcinomas. *OLIG2* mRNA levels were significantly lower in LCNECs than in adenocarcinomas. *MATH3* mRNA levels in LCNECs were significantly higher than in both squamous cell carcinomas and adenocarcinomas.

Conclusion: *INSM1*, *MASH1, OLIG1, OLIG2, MATH2* and *MATH3* are candidate proneural factors that could potentially differentiate lung neuroendocrine carcinomas from non-neuroendocrine carcinomas. In particular, *MATH3* might be an LCNEC-specific factor.

Keywords: Lung; Neuroendocrine carcinoma; Proneural factors; *MATH3*; Non-neuroendocrine carcinoma

Abbreviations SCLC: Small Cell Lung Cancer; LCNEC: Large Cell Neuroendocrine Carcinoma; *INSM1*: Insulinoma-associated Protein 1; NGN: Neurogenin; MASH: Mammalian achaete-scute Homolog; TCF3: Transcription Factor 3; ATOH or MATH: Mammalian Atonal Homolog; NATO3: Nephew of Atonal 3; OLIG: Oligodendrocyte Transcription Factor; NSCL: Neurologic Stem Cell Leukemia

Introduction

The means through which pulmonary neuroendocrine tumors, i.e., carcinoid tumor, small cell lung cancer (SCLC) and large cell neuroendocrine carcinoma (LCNEC) arise and maintain their neuroendocrine features remain undetermined. It is believed that the cells of origin of neuroendocrine carcinomas (SCLC and LCNEC) and non-neuroendocrine carcinomas, e.g. squamous cell carcinoma and adenocarcinoma, both originate from the same endoderm as respiratory epithelium: non-neuroendocrine carcinomas can occasionally be observed in combination with SCLC and/or LCNEC, usually not with carcinoid tumor [1].

To date, 19 proneural factors, which play important roles in fetal neural development, have been discovered in humans: Insulinoma-associated protein 1 (*INSM1*); neurogenin (NGN1); NGN2; NGN3; NEUROD/BETA2; NEUROD2; mammalian achaete-scute homolog (*MASH1*); MASH2; transcription factor 3 (TCF3); mammalian atonal homolog (ATOH1); ATOH7; nephew of atonal 3 (NATO3); oligodendrocyte transcription factor (*OLIG1*); *OLIG2*; OLIG3; mammalian atonal homolog (*MATH2*); *MATH3*; neurologic stem cell leukemia (NSCL1): and NSCL2 [2]. In addition to their *in vivo* roles, NGN1 and/or NGN2 have also been shown to induce neurogenesis in human fibroblasts and human stem cells *in vitro* [3,4]. Some of these proneural factors also play important roles in the development of neuroendocrine cells that originate in the endoderm. For example, *INSM1*, NEUROD/BETA2 and NGN3 are essential for the

differentiation of pancreatic neuroendocrine cells from pancreatic duct cells, which have an endodermal origin [5,6]. The development of gastric endocrine cells also requires *MASH1* and NGN3 in mice [7,8]. With regard to lung neuroendocrine carcinomas, some studies have shown that *MASH1* and/or *INSM1* are related to the development of SCLC [9-12].

At present, there is some controversy as to whether LCNEC is biologically similar to SCLC, and should therefore be treated in a similar fashion to SCLC. A recent study by Rekhtman et al., which employed next-generation sequencing of somatic DNA, demonstrated that lung LCNEC composed of SCLC-like and non-SCLC-like subsets and that LCNEC was thought to be composed of biologically heterogeneous groups [13].

In this study, we investigated whether proneural factors, which might be assumed to be related to the tumorigenesis and maintenance of lung neuroendocrine carcinomas, can be used to differentiate neuroendocrine from non-neuroendocrine lung carcinomas, and possibly SCLC from LCNEC.

Although the somatic DNA genetic profile could provide some key characteristics through which SCLC and LCNEC, and neuroendocrine and non-neuroendocrine carcinomas can be differentiated, protein expression is the key determinant of cell morphology and function. However, it can be difficult to accurately quantify protein expression. For this reason, we have chosen to measure messenger RNA (mRNA) using reverse transcription real-time polymerase chain reaction (PCR) in this study. We have measured and statistically analysed the mRNA expression of all 19 human proneural factors in SCLC, LCNEC invasive squamous cell carcinoma, and invasive adenocarcinoma.

Materials and Methods

We obtained freshly frozen tissue from six pulmonary neuroendocrine carcinomas (3 SCLCs and 3 LCNECs), 10 pulmonary invasive adenocarcinoma and 10 pulmonary invasive squamous cell carcinomas. We choose neuroendocrine carcinomas that were not combined with another histological type. All of the tumors had developed at the periphery of the lung. All of the patients underwent lobectomies with regional lymph node resection and did not receive any preoperative chemotherapy. The tumors were snap-frozen at the time of resection and stored at -80°C until use. The tumor sizes, and patents' age and gender are listed (Table 1).

Total RNA was extracted from snap-frozen samples using the RNeasy Microkit (Qiagen GmbH, Hilden, Germany), RNA was reverse-transcribed to generate complementary DNA using the Primescript II first strand cDNA synthesis kit (Takara Bio Inc, Kusatsu, Shiga, Japan) and intercalating dye-based real-time PCR was performed using Realtime PCR Master Mix (Toyobo Co., Ltd., Osaka, Japan) and an Illumina Eco (Illumina Inc., San Diego, CA, USA). Primers that were specific for each proneural factor were developed using the National Centre for Biotechnology Information gene database; the primer sequences and their annealing temperatures are listed (Table 2). Standard copy numbers for measurement of the mRNA expression were obtained using PCR of complementary DNA through reverse-transcribed RNA of human fetal brain aborted due to intrauterine death at early gestation. The TATA-box binding protein mRNA primer pair was used to normalize for copy number as previously described [14].

Case No.	Sex	Age (yrs.)	Histological Type	Max diameter of tumor
1	Male	56	Small Cell Carcinoma	20 mm
2	Male	64	Small Cell Carcinoma	10 mm
3	Male	68	Small Cell Carcinoma	20 mm
4	Male	76	Large Cell Neuroendocrine Carcinoma	40 mm
5	Male	69	Large Cell Neuroendocrine Carcinoma	40 mm
6	Male	83	Large Cell Neuroendocrine Carcinoma	20 mm
7	Male	83	Squamous Cell Carcinoma	40 mm
8	Female	70	Squamous Cell Carcinoma	50 mm
9	Male	77	Squamous Cell Carcinoma	70 mm
10	Male	76	Squamous Cell Carcinoma	60 mm
11	Male	78	Squamous Cell Carcinoma	60 mm
12	Male	66	Squamous Cell Carcinoma	40 mm
13	Male	59	Squamous Cell Carcinoma	40 mm
14	Male	63	Squamous Cell Carcinoma	40 mm
15	Male	78	Squamous Cell Carcinoma	28 mm
16	Male	82	Squamous Cell Carcinoma	32 mm
17	Female	68	Adenocarcinoma, Acinar Predominance	35 mm
18	Female	44	Adenocarcinoma, Acinar Predominance	20 mm
19	Female	57	Adenocarcinoma, Acinar Predominance	30 mm
20	Male	70	Adenocarcinoma, Acinar Predominance	60 mm
21	Female	74	Adenocarcinoma, Acinar Predominance	35 mm
22	Male	52	Adenocarcinoma, Acinar Predominance	25 mm
23	Female	82	Adenocarcinoma, Acinar Predominance	30 mm
24	Male	82	Adenocarcinoma, Acinar Predominance	40 mm
25	Male	57	Adenocarcinoma, Acinar Predominance	23 mm
26	Female	47	Adenocarcinoma, Acinar Predominance	40 mm

Table 1: Histological types and patient's profiles.

Proneural factor		Primer sequence	Amplicon size	Annealing temperature (°C)
INSM1	Forward	5'-TCTACGAGTGCCATCACTGT-3'	140 bp	60
	Reverse	5'-TCTACGAGTGCCATCACTGT-3'		
NEUROGENIN1	Forward	5'-AGACCTGCATCTCCGACCT-3'	102 bp	63
	Reverse	5'-AGGCTGCCTGTTGGAGTCT-3'		
NEUROGENIN2	Forward	5'-AGGCTGCCTGTTGGAGTCT-3'	115 bp	63
	Reverse	5'-GGCCTTCAGTCTACGGGTCT-3'		
NEUROGENIN3	Forward	5'-CGCAATCGAATGCACAACCT-3'	131 bp	64
	Reverse	5'-GTCAGCGCCCAGCTGTAGTT-3'		
NEUROD/BETA2	Forward	5'-GTTCTCAGGACGAGGAGCAC-3'	164 bp	63
	Reverse	5'-CTTGGGCTTTTGATCGTCAT-3'		
NEUROD2	Forward	5'-CCTCTTGGCTTTAGCTGGTG-3'	128 bp	63
	Reverse	5'-TCGTGTATTTGGATGCCTGA-3'		
MASH1	Forward	5'-ACTGGGACCTGAGTCAATGC-3'	115 bp	64
	Reverse	5'-GCTGTGCGTGTTAGAGGTGA-3'		
MASH2	Forward	5'-GCCCCCACTATCTGGAGTTT-3'	148 bp	63
	Reverse	5'-ACACAGGCTTCTCCCTAGCA-3'		
TCF3	Forward	5'-TAAGCTGCTCTCCCTTGGAA-3'	139 bp	63
	Reverse	5'-GGCAAAGGAGTGAAGGACAG-3'		
ATOH1	Forward	5'-TGAAGGAGTTGGGAGACCAC-3'	110 bp	63
	Reverse	5'-GTAGACGGGATGCTCTCTCG-3'		
ATOH7	Forward	5'-TTCCCCTTTTCTGGGCTACT-3'	121 bp	63
	Reverse	5'-CCGAACAGGACAAACTCACA-3'		
NATO3	Forward	5'-GCCTGGCCATCGTCTATATC-3'	119 bp	63
	Reverse	5'-ACACCCCAGCACTACCAGAC-3'		
OLIG1	Forward	5'-TCCAGTGTTTTGTCGCAGAG-3'	149 bp	63
	Reverse	5'-GCGGTTGGTTTTCGTTTTTA-3'		
OLIG2	Forward	5'-GAAACTACCCCACCGACTCA-3'	113 bp	63
	Reverse	5'-ACCCAAACTGTTTCCACAGC-3'		
OLIG3	Forward	5'-CTTGCGAAGGGACTTTTGAG-3'	141 bp	63
	Reverse	5'-CTGTGGCAAGGACAGAGACA-3'		
MATH2	Forward	5'-AACGACGCTCTGGACAACTT-3'	140 bp	63
	Reverse	5'-TCTGGTCTCTTGCCGATTCT-3'		
MATH3	Forward	5'-AGCTTCATGCCACATTACCC-3'	130 bp	63
	Reverse	5'-TACCATGATGGGGGTAGGAA-3'		
NSCL1	Forward	5'-ATTCCGGATTAGGGGATGAC-3'	106 bp	63

	Reverse	5'-AGAGTGGGCTGAGATGAGGA-3'		
NSCL2	Forward	5'-AGGAGAACCTCGTGGAGACA-3'	104bp	63
	Reverse	5'-GGCGGATGAATGTAGGAGAA-3'		

Table 2: Primer sequences and annealing temperatures for proneural factor amplification.

The Mann-Whitney u-test was used to determine whether there was a statistically significant difference in mRNA levels between SCLC and squamous cell carcinoma; SCLC and adenocarcinoma; LCNEC and squamous cell carcinoma; LCNEC and adenocarcinoma; and squamous cell carcinoma and adenocarcinoma. We were not able to compare SCLC with LCNEC because there were only 3 samples in each group.

This study was approved by the Human Genome and Genetic Analysis Ethics Committee of Showa University (Certification No.232).

Results

Of the 19 proneural factors, the *INSM1*, *MASH1*, *OLIG1*, *OLIG2*, *MATH2* and *MATH3* mRNA copy number was statistically different between histological groups (Figure 1).

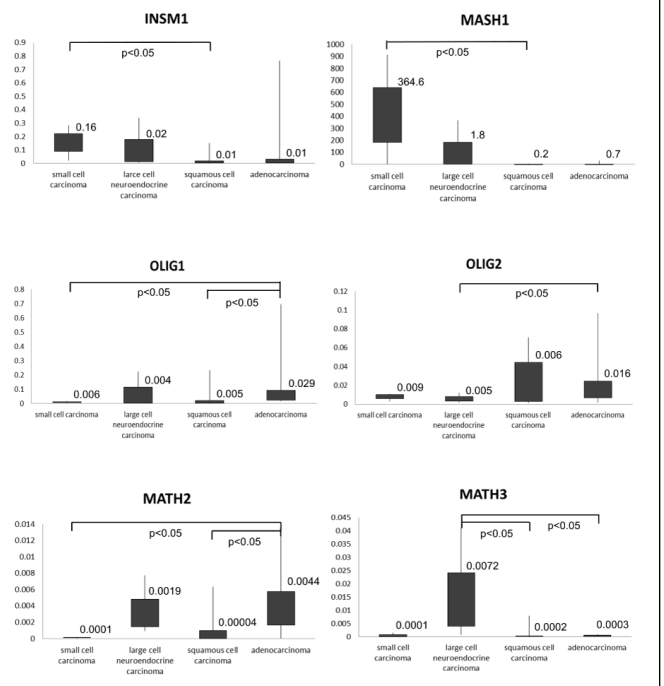

Figure 1: Six proneural factors with significantly different expression in histological groups. Vertical lines show the expression of each proneural factor messenger RNA (mRNA) divided by TATA-box binding protein mRNA expression for normalization. The arabic numerals adjacent to the boxes indicated the median for each histological group. Solid bar and p<0.05 indicate that the difference between two histological types is statistically significant.

INSM1 and *MASH1* mRNA levels tended to be significantly higher in SCLC than in squamous cell carcinoma (p<0.05). The level of *OLIG1* mRNA tended to be significantly lower in SCLC and squamous cell carcinoma than in adenocarcinoma (p<0.05). In LCNEC, the level of *OLIG2* mRNA tended to be significantly lower than in adenocarcinoma (p<0.05). The level of *MATH2* mRNA in SCLC and squamous cell carcinoma tended to be significantly lower than in adenocarcinoma (p<0.05). The *MATH3* mRNA levels in LCNECs tended to be significantly higher than in both squamous cell carcinoma and adenocarcinoma (p<0.05).

Discussion

To date, 19 proneural factors have been discovered, and these are known to play important roles in fetal human neural development. The expression of these proneural factors is controlled in order to regulate neural development [2]. In addition to their roles in neural development, some of the 19 proneural factors also regulate the development of neuroendocrine cells with an endodermal origin [5-9]. For example, transient expression of NGN3 and subsequent expression of *INSM1* and NEUROD/BETA2 initiate the transformation of pancreatic duct cells into neuroendocrine cells [5].

Lung neuroendocrine carcinomas are subdivided into two groups: SCLC and LCNEC. These two carcinomas are thought to be derived from the endoderm, as are the non-neuroendocrine carcinomas, e.g. squamous cell carcinoma and adenocarcinoma; lung neuroendocrine carcinomas that are combined with non-neuroendocrine carcinomas are often observed [1]. Prior to performing this study, we speculated that some proneural factors may play a role in the tumorigenesis and maintenance of lung neuroendocrine carcinomas, and that these factors might be able to differentiate neuroendocrine carcinomas from non-neuroendocrine carcinomas of the lung. However, in this study, we could use a few materials of SCLC and LCNEC because of low incidence of completely removable SCLC that are located peripherally in the lung and low incidence of LCNEC [1,15].

Our results have provided six candidate proneural factors that could be used to differentiate lung neuroendocrine carcinomas from non-neuroendocrine carcinomas: *INSM1*; *MASH1*; *OLIG1*; *OLIG2*; *MATH2*; and *MATH3*. These factors could play an important role in cases of combined neuroendocrine carcinoma with non-neuroendocrine carcinoma. In combined neuroendocrine and non-neuroendocrine carcinoma, *INSM1* and *MASH1* may be involved in SCLC that develops from squamous cell carcinoma, and vice versa; increased *INSM1* and/or *MASH1* expression might induce SCLC formation out of squamous cell carcinoma. Previous reports have shown that *INSM1* and *MASH1* are concerned to SCLC [9-12]. However, in our study, the two proneural factors might merely present the difference between SCLC and squamous cell carcinoma. SCLC that is derived from adenocarcinoma, and vice versa, may be related to *OLIG1* and *MATH2;* decreased *OLIG1* and/or *MATH2* expression could induce SCLC development from adenocarcinoma. Similarly, LCNEC derived from adenocarcinoma, and vice versa, may be related to *OLIG2* expression. Furthermore, *MATH3* expression may be

associated with the differentiation of LCNEC from squamous cell carcinoma or adenocarcinoma, and vice versa.

It is noteworthy that *MATH3* mRNA levels in LCNEC were significantly higher than in either squamous cell carcinoma or adenocarcinoma. Although we were not able to statistically analyze the difference in *MATH3* expression in SCLC and LCNEC, the levels of *MATH3* mRNA seemed to be lower in SCLC than in LCNEC. As such, *MATH3* might be a factor that specifically induces LCNEC tumorigenesis and/or maintenance. However, a recent study by Rekhtman et al. proposed that LCNEC is composed of heterogeneous groups of cells [13]. In this study, we were able to perform our analysis on small number of LCNECs because we used completely resected and fresh-frozen tumor samples to see accurate mRNA copy numbers [16]. To confirm whether *MATH3* expression is a defining characteristic of lung LCNEC, *MATH3* mRNA expression would need to be determined in a larger number of LCNEC cases. It would also be necessary to determine whether the overexpression of *MATH3* mRNA can stimulate LCNEC development in non-tumorous respiratory epithelium or non-neuroendocrine tumor cells *in vivo* and *in vitro*.

The results of our study have also shown that no proneural factors are common to SCLC and LCNEC. This indicates that SCLC and LCNEC differ in their use of proneural factors. Although SCLC and LCNEC both express some common neuroendocrine proteins/antigens, for example CD56, chromogranin A, and synaptophysin [1], some of the proneural factors are most probably specific to either SCLC or LCNEC. Although most advanced stage LCNEC patients are now treated with SCLC chemotherapy regimens, chemotherapy for advanced stage LCNEC is controversial [13,17]. We hope that proneural factors, in particular *MATH3*, might provide a basis for new optional treatments for LCNEC.

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