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Review Article

p73, a sophisticated p53 family member in the cancer world

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p73 belongs to a family of p53-related nuclear transcription factors that includes p53, p73 and p63. The overall structure and sequence homology indicates that a *p63/p73*-like protogene is the ancestral gene, whereas *p53* evolved later in higher organisms. In accordance with their structural similarity, p73 functions in a manner analogous to p53 by inducing tumor cell apoptosis and participating in the cell cycle checkpoint control through transactivating an overlapping set of p53/p73-target genes. In sharp contrast to p53, however, p73 is expressed as two NH_2-terminally distinct isoforms including transcriptionally active (TA) and transcriptionally inactive (ΔN) forms. ΔNp73, which has oncogenic potential, acts in a dominant negative manner against TAp73 as well as p53. p73 is induced to be stabilized in response to a subset of DNA-damaging agents in a way that is distinct from that of p53, and exerts its pro-apoptotic activity. Several lines of evidence suggest that p73 can induce tumor cell apoptosis in a p53-dependent and p53-independent manner. Some tumors exhibit resistance to the p53-dependent apoptotic program, therefore p73, which can induce apoptotic cell death by p53-independent mechanisms, is particularly useful. In this review, we discuss the regulatory mechanisms of p73 activity, and also the functional significance of p73 in the regulation of cellular processes including tumorigenesis, apoptosis and neurogenesis. (*Cancer Sci* 2005; 96: 729–737)

Until recently, the tumor suppressor p53 has been believed to be encoded by a single gene which lacks any structural or functional homologs. The identification of two p53-related proteins, termed p73 and p63, revealed that p53 belongs to a small family of sequence-specific nuclear transcription factors.[1–3] p53 family members share three major functional domains: the NH_2-terminal transactivation domain; the central core sequence-specific DNA-binding domain; and the COOH-terminal oligomerization domain. Of these, the central DNA-binding domain is highly conserved across the family. As expected from their structural similarities, p73 can bind to the p53-responsive elements, and transactivate an overlapping set of p53-target genes implicated in G1/S cell cycle arrest and apoptotic cell death.[1,4] Recent studies demonstrated that p73 is required for p53-dependent apoptosis.[5] Unlike p53, p73 is expressed as at least six variants with different COOH-terminal ends, arising from the alternative splicing at the 3′ portion of the primary transcript.[1,6,7] Each of these splicing variants (TAp73) contains an intact NH_2-terminal transactivation domain, and exerts its transcriptional activity to various degrees. Additionally, *p73* contains a second transcriptional start site within intron 3, giving rise to the NH_2-terminally truncated form of p73 (ΔNp73) which has little transcriptional activity.[8] Similar to p73, ΔNp63 is also generated by an alternative promoter (Fig. 1a,b).[2] ΔNp73 displays dominant negative behavior toward p73 as well as wild-type p53, and has oncogenic potential.[9,10] Of note, we and others found that ΔNp73 is a direct transcriptional target of p73, suggesting that there exists a negative feedback regulation of p73 by ΔNp73, to modulate cell survival and death.[11–13]

Steady-state expression levels of endogenous p73 are kept extremely low under physiological conditions. Similar to p53, p73 is induced to be stabilized at the protein level in response to a subset of DNA-damaging agents, and exerts its pro-apoptotic activity.[14] Accumulating evidence suggests that p73 turnover is regulated through a ubiquitination-dependent and ubiquitination-independent degradation pathway. MDM2 acts as an E3 ubiquitin protein ligase for p53, and promotes the proteasome-mediated proteolytic degradation of p53.[15–17] On the other hand, MDM2 increases the stability of p73,[18] indicating that p73 stability is regulated through a pathway distinct from that of p53. Alternatively, Ohtsuka *et al.* reported that cyclin G binds to p73 and stimulates its proteolytic degradation in a ubiquitination-independent manner, however, the precise molecular mechanism of cyclin G-mediated degradation of p73 remains unknown.[19]

Considering that p73 has a p53-like property and is mapped to the human chromosome 1p36.2-3, a region which is frequently lost in a wide variety of human tumors including neuroblastoma, it is likely that p73 could be one of the classic Knudson-type tumor suppressors.[1] In spite of extensive mutation searches, *p73* was rarely mutated in primary tumors.[20] Additionally, initial genetic studies demonstrated that p73-deficient mice exhibit severe developmental defects, however, they do not develop spontaneous tumors, suggesting that p73 might participate in the regulation of normal development *in vivo*, and that p73 does not link directly to tumor suppression.[5] Indeed, p73 has the ability to induce neuronal differentiation of undifferentiated neuroblastoma cells.[21] However, this viewpoint has been challenged by the observation that mice mutant for p73 and p63 develop spontaneous tumors, and their spectrum is quite different from that of p53-deficient mice.[22] Thus, it is likely that p73 and

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Abbreviations: EEC, ectrodactyly, ectodermal dysplasia, and facial clefts (syndrome); OPC, oligodendrocyte precursor cell; SAM, sterile α motif; YAP, Yes-associated protein.

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As described above, ΔNp73 is transcriptionally inactive due to a lack of the NH_2-terminal transactivation domain in TAp73. However, this viewpoint has been challenged by the recent finding of Liu *et al.* showing that ΔNp73β has weak but distinct transcriptional activity, thereby inducing cell cycle arrest and/or apoptosis.[29] In contrast to ΔNp73β, ΔNp73α failed to induce cell cycle arrest and/or apoptosis under their experimental conditions. According to their results, the NH_2-terminal 13 unique amino acid residues as well as PXXP motifs of ΔNp73β might be a novel activation domain. Thus, it is possible that ΔNp73β might exert a distinct function under certain cellular processes.

Transcriptional regulation of the main promoter of p73

E2F1 transcription factor plays an important role in the regulation of cell cycle progression by inducing the transcription of genes whose products are directly or indirectly required for entry into the S phase.[30] In addition to the proliferative effect of deregulated E2F1 activity, unscheduled E2F1 activation leads to apoptosis to protect cells from cellular transformation.[31] Consistent with this notion, E2F1-deficient mice exhibited a high incidence of unusual tumors.[32,33] E2F1-induced apoptosis is regulated in a p53-dependent or p53-independent manner. It is interesting that the *p73* promoter region contains a TATA-like box and at least three E2F1-binding sites, and indeed the enforced expression of E2F1 strongly stimulates the transcription of *p73* through the direct binding to the E2F1-responsive elements in the *p73* promoter.[34,35] The E2F1-mediated up-regulation of p73 results in a significant induction of apoptosis. Other studies demonstrated that T cell receptor-mediated apoptosis is dependent on both E2F1 and p73.[36] Thus, E2F1-mediated apoptosis requires p73, at least in part. Alternatively, E2F1 might also contribute to the up-regulation of *p73* mRNA levels during muscle and neuronal differentiation of murine C2C12 myoblasts and P19 cells, respectively.[37] It is worth noting that Chk1 and Chk2 are required for the induction of p73 in response to DNA damage, and E2F1 contributes to the Chk kinase-dependent transcriptional regulation of p73.[38] In addition to E2F1, cellular and viral oncogene products such as c-Myc and E1A indirectly activated the transcription of p73.[39]

Recently, Fontemaggi *et al.* identified a 1 kb negative regulatory fragment within the first intron of *p73* gene.[37] Under their experimental conditions, this intronic fragment significantly reduced the activity of the *p73* promoter upon E2F1 overexpression. Of note, the *p73* intronic fragment contained six consensus binding sites for transcriptional repressor ZEB. Ectopic expression of ZEB in C2C12 myoblasts attenuated myotube formation, and repressed the transcription of *p73*. In accordance with these results, the dominant negative form of ZEB had an ability to restore the expression levels of p73 in proliferating cells.

Because DNA hypermethylation contributes to the alteration of the entry of transcription factors into the regulatory region, the epigenetic modification of the *p73* promoter region through aberrant hypermethylation could be an alternative molecular mechanism for silencing the *p73* gene. Corn *et al.* described the aberrant promoter methylation of *p73* as occurring frequently in primary acute lymphoblastic leukemias and Burkitt's lymphomas, whereas the *p73* promoter methylation was not detected in normal lymphocytes or bone marrow.[40] Similar results were also reported by Kawano *et al.*[41] In contrast, hypermethylation of the *p73* promoter region was not observed in solid tumors including breast, renal, colon cancers or neuroblastomas,[42] suggesting that the methylation-dependent silencing of *p73* transcription might be specific to hematological malignancies.

p73 is rarely mutated in human cancers

The *p73* gene has been mapped to human chromosome 1p36.2-3, a region which exhibits frequent loss of heterozygosity in a wide variety of human cancers including neuroblastoma, and its gene product has an ability to promote G1/S cell cycle arrest and/or cell death through apoptosis in certain cancerous cells. Therefore, p73 could act as a tumor suppressor.[1] In spite of an extensive search of the *p73* status in human primary tumors, *p73* was infrequently mutated in many human tumors.[20,43–48] *p73* mutations were detected in fewer than 0.5% of human cancers, whereas over 50% of cancers carry *p53* mutations. For example, only two types of *p73* mutations with amino acid substitution (P405R and P425L) were found in primary neuroblastoma and lung cancer.[20] In addition to the NH_2-terminal transactivation domain, Takada *et al.* found a potential second transactivation domain within the COOH-terminal portion of p73α (amino acid residues 380–513), albeit to a lesser extent than the NH_2-terminal transactivation domain.[49] This region is rich in glutamine and proline residues. Among the two types of p73 mutations, the P425L substitution significantly reduced the transcriptional and growth-suppressive activity of p73α, whereas the P405R substitution had a negligible effect on p73α.[50]

In sharp contrast to p53-deficient mice, which develop tumors with high frequency,[51] p73-deficient mice were viable, but the loss of p73 did not predispose mice to cancer, suggesting that p73 does not function as a classic Knudson-type tumor suppressor,[8] and its possible contribution to tumor suppression is still unclear. Instead, mice lacking p73 displayed severe developmental defects, including hydrocephalus, hippocampal dysgenesis, and abnormalities in the pheromone sensory pathways. These observations strongly suggest that p73 and p53 have distinct biological functions, and p73 plays an important role in normal development, especially in neural development and apoptosis. Because those p73-deficient mice lacked both TAp73 and ΔNp73 variants, further studies of variant-specific knockout mice might provide an insight into the unique role of each variant in tumorigenesis.

As mentioned above, initial genetic studies revealed that p73-deficient mice do not display an increased susceptibility to spontaneous tumorigenesis. More recently, Flores *et al.* examined whether synergistic effects of p73 and p63 could exist, alone or in combination with p53, in tumor suppression.[22] Strikingly, they found that p73 and p63 heterozygous mice ($p73^{+/-}$ and $p63^{+/-}$) developed malignant tumors at high frequency including various tumor types not observed in $p53^{+/-}$ mice, and $p53^{+/-}$; $p73^{+/-}$ and $p53^{+/-}$; $p63^{+/-}$ mice

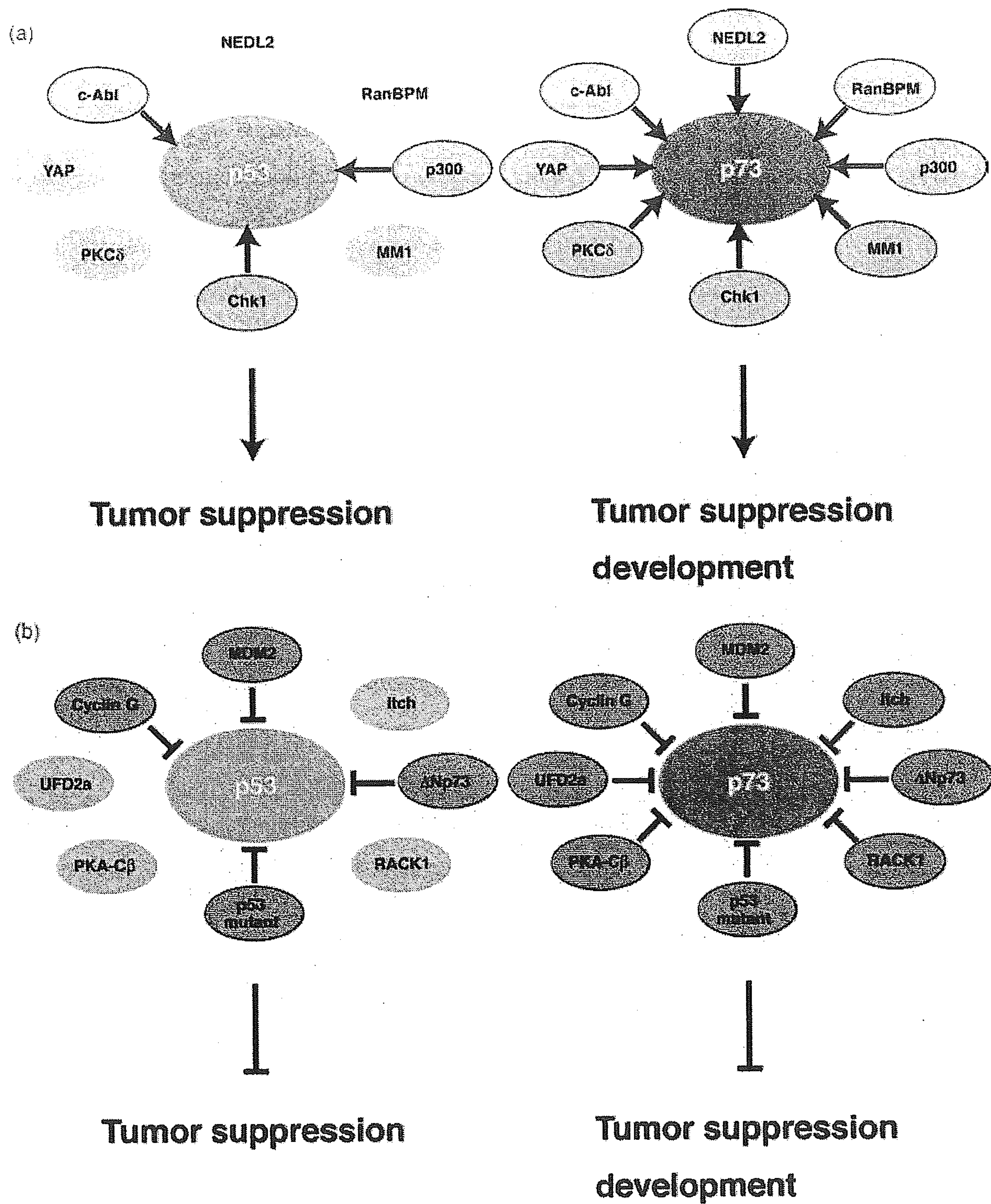

Fig. 4. Positive (a) and negative (b) regulation of p73 or p53 activity through the physical and functional interaction with various cellular proteins. Uncircled proteins have an undetectable effect on p53.

NH_2-terminal transactivation domain, and subsequent proteolytic degradation of p53.[15–17] Similar to p53, MDM2 was also a direct transcriptional target of p73, bound to its NH_2-terminal transactivation domain and thereby inhibiting p73-mediated transcriptional activation and apoptosis. However, MDM2 failed to ubiquitinate p73, and this interaction resulted in an increase in p73 protein stability.[18] Additionally, a newly identified p53-induced E3 ubiquitin

through MDM2 titration.[79] In addition, Goldschneider *et al.* found that p73 promotes the nuclear localization of wild-type p53 in neuroblastoma cells in which p53 is predominantly expressed in cytoplasm.[80] These results suggest that p73 has an ability to enhance the activity of wild-type p53. In contrast, Vikhanskaya *et al.* described that p73 reduces the p53-mediated transcriptional activation through the competition of the same DNA-binding site.[81] These controversial results regarding the effects of p73 on wild-type p53 might be at least in part due to the different cell systems used in those studies.

Recently, it has been shown that p53-dependent apoptosis requires the indirect contribution of at least one other p53 family member, p73 or p63.[5] Thus, it is likely that p73 cooperates with p53 to promote apoptotic cell death. These findings emphasize the functional importance of p73 in the regulation of the DNA damage-induced apoptotic response.

Role of p73 in neuronal differentiation

Considering that p73-deficient mice in which both TAp73 and ΔNp73 have been deleted, displayed profound developmental defects in their nervous and immune systems including a severe distortion of the hippocampal formation, it is likely that p73 contributes to normal neural development.[8] Indeed, ΔNp73 was expressed predominantly in the developing brain and sympathetic neurons, and p53-dependent neuronal apoptosis was inhibited by ΔNp73.[9] Consistent with this notion, De Laurenzi *et al.* demonstrated that p73 is induced to be accumulated during retinoic acid-mediated neuronal differentiation in neuroblastoma cell lines, whereas p53 levels remained unchanged in response to retinoic acid.[21] Under their experimental conditions, ectopic overexpression of p73 in undifferentiated neuroblastoma cell lines resulted in the induction of neurite extension as well as the expression of neuronal differentiation markers. In contrast, the transcriptionally inactive mutant form of p73 had undetectable effects on the neuronal differentiation. Similar results were also observed during neuronal differentiation in P19 cells exposed to retinoic acid.[46] Of note, Billon *et al.* described that the ectopic expression of p73 induces oligodendrocyte precursor cell (OPC) differentiation, and that ΔNp73 inhibits OPC differentiation in culture.[82] These observations strongly suggest that, in addition to its apoptosis-inducing activity upon DNA damage, p73 plays a pivotal role in the regulation of proper neuronal differentiation.

Role of p73 in the p53-independent cellular pathway

p53 plays a central role in the regulation of apoptotic cell death in response to DNA damaging agents. p53 function is lost by various mechanisms, including loss of function mutations within the *p53* gene itself or defects in upstream and/or downstream mediators of p53. Recent findings clearly demonstrated that p53-dependent apoptosis in response to DNA damage is impaired in cells lacking both p73 and p63, indicating that p73 and p63 are critical components of the apoptotic response to DNA damage.[5] Accumulating evidence strongly suggests that the pro-apoptotic activity of p73 is regulated through a pathway distinct from that used for p53. As certain cancerous cells were resistant to p53-dependent apoptotic cell death, p73 could be one of several candidate tumor suppressor proteins which can promote apoptosis by p53-independent mechanisms. Previous studies revealed that the exogenous expression of p73 in p53-deficient cells results in significant cell death through apoptosis in a p53-independent manner.[4] It is worth noting that p73 has an ability to promote apoptotic cell death in various pancreatic cells lacking functional p53, which are resistant to wild-type p53 gene transfer.[83] Thus, it is possible that p73 could be particularly useful in treating cancerous cells with non-functional p53.

What is the difference between p73 and p63?

The overall genomic organization of *p63* is quite similar to that of *p73*; the *p63* gene contains 15 exons.[23] Although *p63* is mapped to human chromosome 3q27–29, a region which is altered in a variety of cancers derived from lung, cervix or ovary, *p63* was infrequently mutated in primary tumors.[2,20] Like p73, p63 gives rise to at least six splicing variants as well as an NH_2-terminally truncated form of p63 (ΔNp63) arising from the alternative promoter usage. ΔNp63, a direct transcriptional target of p53, had a dominant negative effect on TAp63.[84] As expected from its structural similarity to p73, p63 can bind to the p53-responsive element and transactivate an overlapping set of p53-regulated genes, thereby inducing cell cycle arrest and/or apoptosis.[2] In contrast to p73, E2F1 did not stimulate the transcription of *p63*, although the putative E2F1-binding sites were found within the *p63* promoter region.[84]

At a sequence level, *p63* is much more similar to *p73* than *p53*, raising the possibility that *p73* or *p63* might be the original *p53* family gene, and that *p53* might be phylogenetically younger.[85] In support of this notion, p73 and p63 contribute to normal development, and p53 holds additional biological properties such as strong tumor suppressor activity. Despite the structural and functional similarities between p73 and p63, knockout phenotypes and the expression patterns of p63 were quite different from those of p73. In sharp contrast to *p73*, whose expression was restricted to the epidermis, sinuses, inner ear and brain, *p63* was predominantly expressed in the epidermis, cervix, urothelium and prostate.[23] Unlike p73-deficient mice, p63-deficient mice exhibited severe defects in limb, cranio-facial and epithelial development.[86,87] For example, p63-deficient mice lacked all squamous epithelia, and displayed severe forelimb truncations. Consistent with these developmental defects, *p63* mutations were detected in children affected by EEC (ectrodactyly, ectodermal dysplasia, and facial clefts) syndrome.[88] Thus, it is likely that p73 and p63 have overlapping and distinct biological activities, and they express their specific functions depending on their unique sites of action.

Conclusion

p53 and p73 share extensive structural and functional similarities. They have overlapping as well as distinct biological functions. In addition to its potent tumor suppressor function, at least in specific tissues, p73 plays a pivotal role in normal neurogenesis *in vivo*. Similar to

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Profile of neuroblastoma detected by mass screening, resected after observation without treatment: results of the Wait and See pilot study

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Index words:
Neuroblastoma;
Mass screening;
Spontaneous regression;
Observation

Abstract

Background/Purpose: Neuroblastoma (NB) detected by mass screening (MS) usually shows favorable prognosis and sometimes regresses spontaneously. Therefore, the authors started an observation program for these patients to avoid overtreatment. In this study, the authors analyzed the profile of NB resected after observation to elucidate the nature of NB detected by MS.

Methods: Between 1994 and 2004, 22 NB patients matched the following criteria and entered the observation program after obtaining informed consent: stage I or II, less than 5 cm in diameter, and without involvement of large vessels or organs. If increase in size, elevation of tumor markers, or evidence of metastasis was observed, the tumor was immediately resected.

Results: Thirteen (59%) of 22 cases showed spontaneous regression. In the remaining 9 cases, tumors were resected because of parents' request, increase in size, and/or elevation of tumor markers. Four tumors had at least one unfavorable biologic feature, and 3 of them had more than 2. According to Shimada's system, 2 had unfavorable histology. One was diploid tumor, 3 had 1p deletion, and Trk-A expression was low in 4 tumors. All patients survived without evidence of recurrence.

Conclusions: The observation program has shown that at least one third of the NB detected by MS regressed spontaneously. On the other hand, MS may detect some cases with unfavorable tumor in early stage, which benefit from screening.
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The prognosis of neuroblastoma (NB) in younger than 1 year is much better than that older than 1 year. In 1985, Japanese nationwide mass screening (MS) using urinary vanillyl mandelic acid (VMA) and homovanillic acid (HVA) for 6-month-old infants was started to improve the prognosis of this tumor [1]. Since then, more than 2000 patients with NBs were discovered and treated. Their prognosis was extremely good: more than 97% of them are alive [2]. Moreover, the majority of the NBs in MS group were biologically favorable even in advanced stages [3]. Recent reports have also shown that NB detected by MS sometimes regresses spontaneously [4,5]. Although the incidence of NB has remarkably increased after introduction of MS, several reports suggest that the number of advanced NB patients older than 1 year has not decreased substantially [3]. These findings indicate that MS has detected tumors that otherwise may have regressed spontaneously without recognition [4,5]. Therefore, we started an observation program in the limited cases to avoid overtreatment and to estimate how frequently regression occurs (Wait and See pilot study) in June 1994 [6]. Our preliminary results have shown that at least 60% of NB cases who entered our observation program regressed spontaneously [6]. In this study, we analyzed the profile of NB resected after observation to elucidate the nature of NB detected by MS.

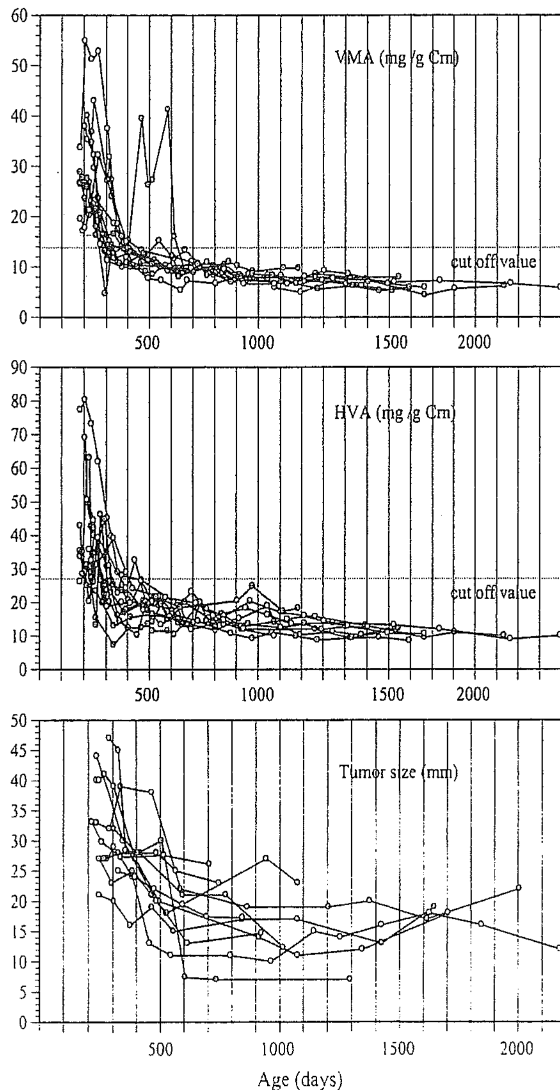

Fig. 1 Changes in levels of VMA and HVA, and tumor size in regressed cases.

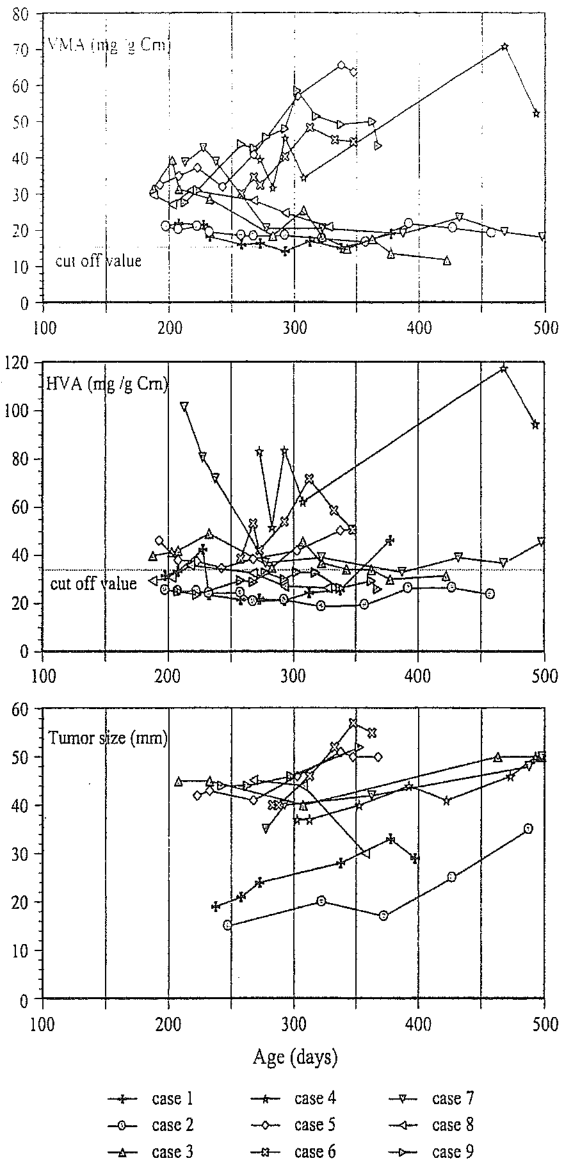

Fig. 2 Changes in levels of VMA and HVA, and tumor size in regressed cases. The cutoff values of urinary VMA/HVA levels were 12.6 and 27.0 mg/g Cm, respectively.

1. Patients and methods

The details of our observation program have been described previously [6]. The entry criteria were as follows: (1) stage I and II [7], (2) less than 5 cm in diameter, (3) no involvement of large vessels or organs and surgical resection is not difficult, and (4) parents' informed consent. The patients were strictly followed up without receiving any treatment. If increase in size, elevation of tumor markers, or evidence of metastasis was observed, the tumor was

Table 1 Neuroblastoma resected after observation

Case	Stage	Origin	Initial			Preoperative			Reason of interruption	Obs days	Age at ope (mo)
			VMA	HVA	Size	VMA	HVA	Size			
1	IVs	Lt retro	21.1	31.6	19	19.0	46.3	33	Size ↑, marker ↑	199	13
2	I	Lt retro	21.0	25.5	15	19.1	23.7	25	Size ↑, parents' request ↑	291	16
3	I	Lt adrenal	31.5	39.9	40	11.7	31.5	50	Size ↑	309	16
4	I→III	Lt adrenal	39.5	83.3	37	52.4	94.5	50	Size ↑, LN meta ↑	226	16
5	I→II	Rt adrenal	32.5	46.3	42	63.7	50.9	50	Size ↑, marker ↑	174	11
6	I	Lt adrenal	30.0	38.7	40	44.4	50.6	57	Size ↑, marker ↑	96	11
7	II	Rt retro	38.6	101.5	35	18.0	45.4	50	Size ↑	300	16
8	I	Rt adrenal	29.7	29.5	45	20.9	26.7	40	Parents' request	172	11
9	I→III	Rt retro	27.6	25.0	44	43.3	26.0	52	Size ↑	159	12

Size indicates longer diameter (mm); stage, Evans' staging system [7]; Lt, left; Rt, right; retro, retroperitoneum; LN meta, lymph node metastasis; obs, observation; ope, operation.

resected immediately. Biological features of the tumor were identified: Shimada's histological features [7], amplification of N-*myc* (Southern blotting, SRL Inc, Tokyo, Japan), 1p deletion (FISH, Otsuka Assay Laboratory, Tokyo, Japan), and expression of Trk-A (immunohistochemistry) [8]. Between June 1994 and March 2004, 43 patients with NB detected by MS were admitted to our hospital, and 22 (51 %) of them matched the criteria and were enrolled in the observation program. They included 11 boys and 11 girls. Age at diagnosis ranged from 7 to 11 months. Origins of the tumors were adrenal grand in 15 and retroperitoneal paraganglion in 7. Seventeen cases were in stage I, and 3 in stage II. Two patients in stage IV were also enrolled in the study, because the parents refused any treatment.

2. Results

Thirteen (59%) of 22 cases showed spontaneous regression and continued the observation program. The observation periods ranged from 15 to 116 months until May 2004. Urinary levels of VMA and HVA decreased and normalized within 18 months in all cases except one (Fig. 1). The tumor size (maximum diameter) was decreased to 0.7 to 2 cm within 3 years in all the cases; however, no tumor has disappeared (Fig. 1). In the remaining 9 (41%) cases, tumor resection was performed in 96 to 309 days after initial screening. In 2 cases, parents decided to stop the observation; therefore, the tumors were resected after 291- and 172-day observation. The remaining 7 tumors were resected because of increase in size and/or elevation of tumor markers (Fig. 2). In 2 cases (cases 4 and 5), lymph node metastases were observed at operation, resulting in the upgrading of the tumor stage from I to II and I to III, respectively. In case 9, tumor growth resulted in the upgrading of stage from I to III. The profile of the resected cases is listed in Tables 1 and 2. Four tumors (cases 2, 3, 7, and 8) showed differentiation to ganglioneuroblastoma. Biological features of the 9 resected tumors were listed in Table 2. According to Shimada's system, 7 were favorable, and 2 were unfavorable. DNA content was aneuploid in 7 tumors and diploid in one. Three of them had 1p deletion. Trk-A expression was low in 4 cases. Two cases (cases 6 and 7) showed biological heterogeneity in the tumor. No tumor showed N-*myc* amplification. Four (44%) tumors had at least one unfavorable biologic feature, and 3 of 4 tumors had more than 2 features. All tumors were resected completely without major surgical complication. Two of

Table 2 Neuroblastoma resected after observation

Case	His	Shimada's classification	N-*myc* (copy)	DNA ploidy	1p deletion	Trk-A expression	Operative procedure	Post operative chemotherapy
1	NB	Unfavorable	1	A	–	Low	Open	+
2	GNB	Favorable	1	A	–	High	Open	–
3	GNB	Favorable	1	ND	–	High	Laparoscopic	–
4	NB	Favorable	1	A	–	High	Open	+
5	NB	Favorable	1	A	+	Low	Open	–
6*	NB	Favorable	1	A/D	+	High/low	Open	–
7*	GNB	Unfavorable	1	A/A	+/–	Low/low	Open	+
8	GNB	Favorable	1	A	ND	ND	Laparoscopic	–
9	NB	Favorable	1	A	–	ND	Open	+

His indicates histology; GNB, ganglioneuroblastoma; ND, not detected; A, aneuploid; D, diploid.
* Showed intratumoral heterogeneity.

them were resected laparoscopically. Postoperative chemotherapy was performed in 4 cases who had stage III tumor and/or unfavorable prognostic factors. All patients survived without evidence of recurrence.

3. Discussion

In the presented series, among 43 NBs detected by MS, 22 have entered the observation program, and 13 tumors regressed spontaneously. Therefore, tumors are expected to regress at least in 59% of the patients who fulfilled our observation program or in 29% of all the patients detected by MS. Moreover, 5 of the 9 resected tumors had favorable biologic feature, which indicate that they have possibility of spontaneous regression if observation was continued. Therefore, the incidence of spontaneous regression should be higher. Similar observation trials were made at other institutes; Nishihira et al [5] reported that 17 (65%) tumors of 26 patients who were enrolled in their observation program regressed. Yamamoto et al [4] reported that 92% of the observed tumors or 44% of all the tumors identified by MS have spontaneously regressed. These results suggested that at least one third of the NB detected by MS might regress spontaneously. To avoid the overtreatment, several trials have been introduced including laparoscopic surgery [9] and reduction of chemotherapy [5]. We consider that observation program is most effective to avoid the unnecessary treatment.

Most of screened NBs were biologically favorable. However, there was a small group with biologically unfavorable factors, such as diploid DNA content, chromosomal pattern with 1p deletion, lower Trk-A expression, and N-*myc* amplification. Suita et al reported that 5 of 285 NBs detected by MS had amplified N-*myc* oncogene, 4 of 74 showed unfavorable Shimada's histological findings, and 3 of 33 had an unfavorable DNA ploidy pattern [3]. Therefore, MS may detect 2 biologically different groups, described as favorable and unfavorable. The unfavorable tumors may progress and/or disseminate in the future. Mass screening enables the cases with unfavorable tumor to undergo early treatment; therefore, these cases may benefit from MS. The favorable and unfavorable group can be identified only after analysis for the surgically removed tumor specimens. Therefore, identification of new prognostic factors that could distinguish the regressing tumor without surgery is required. Our examinations of the resected tumors after observation revealed high incidence of unfavorable biologic factors and upgrading of the tumor stage. Four (44%) of 9 resected tumors had at least one unfavorable biological feature. This percentage is much higher than reported percentage (0% to 20%) among all of the NBs detected by MS [3,10]. These findings suggest that unfavorable tumor may grow in size; therefore, they can be distinguished from the regressing tumors and be resected if they enter the observation program.

To clarify the benefits of MS in public health, we should prove whether MS decreases the number of advanced tumors and improves the survival. Several reports suggested that MS did not improve the overall mortality rate of NB [3]. Moreover, the results of the present study have proven that MS detects a considerable number of regressing tumors. Treatment of the regressing tumors may extremely harm the significance and cost benefit of MS, because the patients with regressing tumors had not received any treatment if MS would not have detected them. Based on the information obtained so far, the MS for NB at 6 months of age probably should not contribute to the public health. Therefore, Japanese Ministry of Health and Welfare has decided to discontinue the MS for NB at 6 months of age in April 2004. At the same time, our observation program at 6 months of age was also closed.

The optimal time for MS should be the point at which NB regressing spontaneously can no longer be detected, but more aggressive tumors can be found. Our observation program may determine the optimal timing of screening. Urinary VMA and HVA levels were normalized within 18 months in most of the regressed cases (Fig. 1). On the other hand, in the patients whose tumor increased in size, VMA and HVA levels were over the cutoff value at the time of operation. These findings suggest that screening in age around 18 months may be more effective; most of the favorable tumor has regressed, and unfavorable tumor should be detected. Now we are planning to start the new MS program for 18-month-old infants in Osaka prefecture, expecting to clarify whether MS in older age has benefit or not.

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原著

神経芽腫の予後、とくにDNA ploidyによるタイプ分類および組織型との関連性

Prognosis of neuroblastoma in relation to the histological classification and the DNA ploidy type

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要旨
神経芽腫，308例の予後と組織学的分類，DNA ploidyとの関連性を検索し，組織型やタイプ分類にかかわらず1.5歳未満症例の予後は良好であり，組織型が予後不良群ではDNA倍数体，年齢にかかわらず予後は不良との結果が得られた．タイプ2神経芽腫は予後良好のタイプ1と予後不良のタイプ3の中間に位置し，多様な組織型，臨床像を示し，そのなかでも予後不良群の早期発見は予後改善に有用であることが示唆された．
Key words：**神経芽腫，INPC組織型，DNA倍数体 タイプ分類**
neuroblastoma，histological classification(INPC)，DNA ploidy typing

はじめに

神経芽腫のなかには自然退縮して腫瘍縮小し，あるいは分化・成熟傾向を示して神経節腫などの良性腫瘍に移行し，予後良好な経過を示す例がある．とくに本邦でのマススクリーニング（マス）で発見される乳児期の神経芽腫は上記のような特異な生物学的特性を示す．一方で，腫瘍浸潤，増殖傾向が著しく，骨髄をはじめ，多臓器への腫瘍進展，転移を示し，治療に抵抗性のきわめて予後不良の腫瘍も乳児期に存在するので，予後不良例を早期発見することが神経芽腫例の予後向上には重要な点である．

この神経芽腫の多様性は腫瘍のもつ生物学的特性に由来すると考えられ，この特性のひとつとして，腫瘍細胞核のDNA量解析を利用したDNA倍数体による神経芽腫のタイプ分類（タイプ1～3）の検索から，タイプ2腫瘍が1歳以降に多く，タイプ1とタイプ3の中間型の臨床的予後を示すとされている[1]．とくに本邦ではこのようなタイプ2神経芽腫が1歳以下でもマスで発見される頻度は高いと考えられる．また，タイプ2神経芽腫が腫瘍進展して予後不良例となる可能性もあり，マス発見のターゲットになる症例でもあることが示唆される．今回の調査研究では，小児医療3施設の神経芽腫，308例の組織学的分類と年齢，臨床的予後との関連性，および291例のDNA倍数

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表 1　International Neuroblastoma Pathology Classification (INPC)[2,3]

Neuroblastoma : undifferentiated
poorly differentiated
differentiating
Ganglioneuroblastoma : intermixed
Ganglioneuroma : maturing
mature
Ganglioneuroblastoma : nodular, favorable
nodular, unfavorable

<u>Favorable group</u>
- poorly differentiated NB (<1.5 yrs, low or intermediate MKI)
- differentiating NB (<1.5 yrs, low or intermediate MKI)
- differentiating NB (1.5 – 5 yrs, low MKI)
- GNB intermixed / GN (in any age)
- GNB nodular (favorable NB components)

<u>Unfavorable group</u>
- undifferentiated NB (in any age)
- high MKI tumors (in any age)
- neuroblastoma (1.5 – 5 yrs) :
  - poorly differentiated
  - differentiating (intermediate MKI)
- all neuroblastoma (5 yrs≦)
- GNB nodular (unfavorable NB components)

Groups are modified

体によるタイプ分類と予後との関連性を検討し，とくにタイプ 2 神経芽腫の性格を明確にし，今後のマスの評価と方法の改善に役立てることを目的とした．

症例および方法

1. 症　例

埼玉県立小児医療センター(1983-2002 年)，神奈川県立こども医療センター(1990-2003 年)，静岡県立こども病院(1977-1999 年)の神経芽腫，計 308 症例（タイプ分類は 291 例で施行）を対象とした．

2. 臨床所見等の調査項目

症例の生年月日，診断の経路，マス受診の有無，原発部位，病期，治療，転帰，死亡日を調査項目とした．なお，年齢は腫瘍切除時点を基本と，1.5 歳未満，1.5 ～ 5 歳，5 歳以上に分類して検討した．検査項目としては尿中 VMA ／ HVA，LDH，NSE，フェリチン，MYCN 増幅の有無なども調査し，DNA 倍数体によりタイプ分類をおこない，各タイプ別に組織型を含めた予後との関連性を検討した．DNA 倍数体は腫瘍組織の新鮮材料あるいはパラフィン包埋組織を用い，フローサイトメトリーにより腫瘍細胞核の DNA 量を測定し，正常倍数体を指標とした腫瘍組織の倍数体および DNA index (DI) を解析した．倍数体による症例のタイプ分類として，タイプ 1は MYCN 非増幅で，DI が 1.2 から 1.8 までの 3 倍体あるいは 2.2 以上の 5 倍体を示す例（hyperdiploid, near triploid)，タイプ 2 は DI が 1.2 未満の 2 倍体あるいは 1.8 から 2.2 の 4 倍体で (near diploid, near tetraploid)，MYCN の非増幅例，タイプ 3 は 2 倍体あるいは 4 倍体 (near diploid, near tetraploid) に加えて MYCN 増幅例とした[1]．

3. 組織学的検索

INPC 国際分類 (International Neuroblastoma

表2　腫瘍組織型と1.5歳未満，1.5歳以上症例の臨床的予後との関連性

倍数体	<1.5歳（生存数）	<1.5歳（死亡数）	1.5歳≦（生存数）	1.5歳≦（死亡数）	計（生存数）	（死亡数）
2倍体						
予後良好群	64	4	12	3	76	7
予後不良群	8	3	15	17	23	20
3倍体						
予後良好群	142	5	12	0	154	5
予後不良群	4	2	3	6	7	8
多倍数体						
予後良好群	8	0	0	0	8	0
予後不良群	0	0	0	0	0	0

Pathology Classification）により腫瘍の組織型を分類し，年齢などを加味した予後良好群，予後不良群についてもその基準により分類し（表1），臨床的所見と腫瘍の組織学的診断・組織型との関連性について調査，検討した．

検討結果

Ⅰ．組織型およびDNA ploidyと臨床的予後との関連性

1．予後良好の組織型群

3倍体／5倍体，および2倍体／4倍体において，予後良好の組織型群では，1.5歳未満，1.5歳以上例のいずれも全体として臨床的予後は良好であった（表2）．すなわち，1）2倍体の生存率は89.4％であり，1.5歳未満例の生存率が94.1％，1.5歳以上は80.0％であり，2倍体では1.5歳未満例の方が1.5歳以上例よりも予後良好であった．2）3倍体症例の全体としての生存率は96.9％であった（1.5歳未満は96.6％，1.5歳以上は100％）．なお，多倍数体あるいは複数病変が存在した8症例はいずれも1.5歳以下で，予後は良好であった．

2．予後不良の組織型群

2倍体，3倍体例，あるいは年齢にかかわらず，組織型が予後不良群では，予後良好群よりも全体として死亡率が高いが，年齢的要素が関与している．すなわち，1）2倍体症例全体としての生存率は53.5％であるが，1.5歳未満の生存率は72.7％，1.5歳以上が46.9％であり，2倍体の1.5歳以上例では生存率が低くなる．2）予後不良組織型での3倍体症例の全体としての生存率は46.7％であるが，1.5歳未満例が66.7％に対して1.5歳以上は33.3％と生存率が低くなっている．

3．全体のまとめ

神経芽腫308症例の予後と組織型，ploidyとの関係では，1.5歳未満例の神経芽腫の予後は全体として良好であった．すなわち，1）症例全体の生存率（overall survival）は86.5％であった．2）1.5歳未満例と1.5歳以上例の予後については，1.5歳未満例が94.2％，1.5歳以上例が61.8％であり，統計学的にカイ二乗検定で年齢的因子と死亡率の間には関連性のあることが認められた（危険率1％）．

Ⅱ．神経芽腫のタイプ分類と組織型および予後との関連性

1．タイプ 1 症例

3倍体／5倍体のタイプ1症例，171例では，年齢分布は1.5歳までが主体であり，組織型は比較的未熟な神経芽細胞からなる腫瘍像を示し，多く（92.4％）は予後良好群の範疇に入る（表3）．タイプ1におけるマス例の比率は89.5％である．タイプ1のマス例，非マス例のいずれも1.5歳未満の予後良好例の比率がきわめて高いが，1.5歳以上，とくに5歳以上では予後不良群の比率が高くなる．ただ，1.5歳～5歳，5歳以上でもマス

表3　神経芽腫のDNA倍数体タイプと組織型・予後との関連性―タイプ1症例―

組織型	1.5歳未満（症例数）	1.5−5歳（症例数）	5歳以上（症例数）
NB, undifferentiated	−	−	−
NB, poorly differentiated	126	3	4
NB, differentiating	27	2	1
GNB, intermixed	5	3	−
GN, maturing/mature	−	−	−
GNB, nodular, favorable	−	−	−
GNB, nodular, unfavorable	−	−	−
予後良好群数／予後不良群数	152／6	5／3	1／4
マス例数／非マス数例	145／13	6／2	2／3

NB: neuroblastoma, GNB: ganglioneuroblastoma, GN: ganglioneuroma

表4　神経芽腫のDNA倍数体タイプと組織型・予後との関連性―タイプ2症例―

組織型	1.5歳未満（症例数）	1.5−5歳（症例数）	5歳以上（症例数）
NB, undifferentiated	−	−	−
NB, poorly differentiated	47	12	5
NB, differentiating	11	3	1
GNB, intermixed	3	4	−
GN, maturing/mature	−	4	−
GNB, nodular, favorable	1	−	−
GNB, nodular, unfavorable	−	2	1
予後良好群数／予後不良群数	60／2	11／14	0／7
マス例数／非マス数例	54／8	18／7	1／6

NB: neuroblastoma, GNB: ganglioneuroblastoma, GN: ganglioneuroma

表5　神経芽腫のDNA倍数体タイプと組織型・予後との関連性―タイプ3症例―

組織型	1.5歳未満（症例数）	1.5−5歳（症例数）	5歳以上（症例数）
NB, undifferentiated	2	2	2
NB, poorly differentiated	6	9	2
NB, differentiating	1	1	−
GNB, intermixed	−	1	−
GN, maturing/mature	−	−	−
GNB, nodular, favorable	−	−	−
GNB, nodular, unfavorable	−	−	−
予後良好群数／予後不良群数	2／7	2／11	0／4
マス例数／非マス数例	5／4	8／5	0／4

NB: neuroblastoma, GNB: ganglioneuroblastoma, GN: ganglioneuroma

例は存在した.

2. タイプ 2 症例

2倍体/4倍体でMYCN非増幅のタイプ2症例, 94例では, 年齢分布は1.5歳までを主体とし, タイプ 1よりも広く年齢分布し, 未熟な神経芽細胞から, 比較的, 分化した神経節細胞からなる組織像まで種々の所見を示した(表4). 1.5歳未満では予後良好群の範疇に入るが(96.8%), 1.5歳以降, とくに5歳以降は予後不良群の比率が高くなっている. マスで発見される腫瘍ではあるが, 腫瘍退縮・成熟像, あるいは腫瘍進展など, 症例に多様性がみられる. タイプ2のマス例の比率は全体として77.7%で, 1.5歳未満では87.1%, 1.5～5歳では72.0%, 5歳以上では14.3%であった.

3. タイプ 3 症例

2倍体/4倍体でMYCN増幅を示すタイプ3症例, 26例では, 年齢分布は1.5～5歳を主体とし, タイプ 2よりも1.5歳以上の年齢層に広く分布している(表5). 組織型では未分化・未熟な神経芽細胞からなる腫瘍像を示し, 多く(84.6%)は予後不良群の範疇に入り, タイプ3のマス例の比率は全体として50%であり, マス発見が困難な腫瘍である. また, 1.5歳未満でも予後不良群例が多く, 1.5～5歳例, および5歳以降ではきわめて予後不良例が多くなる. マス発見例との関連では, 1.5歳未満の予後不良群7例中の5例はマス例である. 1.5～5歳の予後不良群11例中の6例がマス発見例で, 5歳以降はすべて非マス例である.

考　察

3施設の神経芽腫, 308例を対象として, INPC国際分類によりその組織型を分類し[2-4], 腫瘍の細胞核DNA量解析などから倍数体により腫瘍型を3タイプに分類し[1], 予後などの臨床所見を含めて, 組織型とDNA ploidyおよび各タイプとの関連性について検討した.

この結果を組織型の面からみると, 組織型にかかわらず全体として1.5歳未満の神経芽腫例の臨床的予後は良好である. 予後良好群の組織型の中でも3倍体症例はとくに予後良好であり, また, 2倍体, 4倍体症例であっても1.5歳未満例は予後良好である. 組織型が予後不良群においては, DNA倍数体あるいは年齢にかかわらず臨床的にも予後は不良である. また, DNA ploidyによるタイプ分類からみると, タイプ1症例の年齢分布は1.5歳までを主体とし, 組織型の多くは予後良好群であり, マスで発見される腫瘍としての特徴を示す. タイプ3の症例は, タイプ1, 2のように1.5歳以下に偏ることなく, 1.5～5歳, 5歳以上まで広く年齢分布し, 未分化・未熟な神経芽細胞からなる腫瘍像を示す例が多い. すなわち, タイプ3症例の大半は年齢にかかわりなく予後不良群の範疇に入り, また, 臨床的stageも高く, マスでの発見よりも臨床的に診断されることの多い腫瘍である. 今回の多数例の検索により, タイプ2に分類される症例はタイプ1とタイプ3症例の中間に位置することが明らかとなったが, タイプ2症例はタイプ1症例よりも広く年齢分布し, 未熟神経芽細胞ないし分化・成熟した神経節細胞からなる種々の組織型を示す. なかでも1.5歳未満のタイプ2症例の多くは予後良好群であり, 1.5歳～ 5歳では予後不良群の比率がやや高くなり, 種々の組織型を示す. 一方, 5歳以上の場合はタイプ3症例とほぼ同様である. タイプ2症例はマスで発見される頻度の高い腫瘍ではあるが, 腫瘍退縮, 成熟, 腫瘍進展例など, 腫瘍像や臨床的予後には多様性がみられる.

神経芽腫には元来, その腫瘍組織像の多様性があり, とくにマススクリーニングで発見される1歳未満例の腫瘍では腫瘍部位により腫瘍組織像が異なり, あるいは年齢経過とともに腫瘍細胞の変性, 退縮, 消失などの所見が少なからず認められる[5,6]. 一方では腫瘍の分化・成熟傾向を示す症例もあり, 無治療自然経過観察中に腫瘍摘出した症例の検索からも, 1歳を超えると腫瘍中の神経芽細胞が神経節細胞に成熟し, とくに1.5歳以降には神経芽腫が神経節腫へ移行していく成熟過程が組織学的にも窺える[7]. 症例によっては退縮像, 成熟像が混在する症例, あるいは未熟な成分が腫瘍結節として残存する症例も少なくない.

このような多様な生物学的, 形態学的腫瘍性格をもつ神経芽腫については, マスをはじめとして,

種々の方向からの検索がおこなわれ，その腫瘍組織の生物学的および形態学的特徴が少しずつ解明されつつある．マスの観点からは，タイプ２の1.5歳未満のマス発見例は87.1％，1.5～5歳では72.0％と多く，タイプ３でも1.5歳未満，1.5～5歳までは半数以上がマス例であるが，５歳以降にはマス発見例はなく，そして1.5歳以降には予後不良群の頻度が高くなることから，それらをマスやその他の方法で早期に発見し，早期に治療することが今後，神経芽腫の臨床的予後を改善するものと期待される．

おわりに

神経芽腫の組織学的分類とタイプ分類，臨床所見との関連性を検討した結果，従来，１歳以降に多く発生し，タイプ１とタイプ３の中間型の予後を示すとされたタイプ２が本邦ではマス発見例にも多くみられることが分かった．このタイプ２症例の早期発見を含め，乳児期の神経芽腫の治療，臨床的予後などの検討は今後もさらに必要となろう．

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ORIGINAL ARTICLE

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Glutamine regulates amino acid transport and glutathione levels in a human neuroblastoma cell line

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Abstract Both amino acid transport and glutathione play a key role in regulating cancer cell growth. Glutamine can serve as an important ATP source for cancer cells, and it can supply glutamate, a precursor for the synthesis of glutathione, by the hydrolysis of glutamine. We examined the effects of glutamine concentrations [2 mM (control), 400 μM, 200 μM, and 0 μM] on cell growth, amino acid transport, and glutathione levels in a human neuroblastoma cell line, SK-N-SH, by using cell culture technique. Cell growth rates were dependent on glutamine concentrations in culture media. Glutamate transport significantly increased in glutamine-deprived groups, and this increase was remarkable in lower glutamine groups (200 μM and 0 μM glutamine). Glutamine deprivation resulted in a significant decrease in glutathione levels by 20% compared with control, but glutathione in 0 μM glutamine was maintained with the same levels found in 400 μM and 200 μM glutamine. DNA and protein synthesis correlated directly with glutamine concentrations in culture media. Our results suggest that glutamine mediates neuroblastoma cell proliferation by regulating amino acid transport and glutathione synthesis, both when sufficient nutrients are present and when key nutrients such as glutamine are in limited supply.

Keywords Glutamine · Amino acid transport · Glutathione · Neuroblastoma

Introduction

Malignant cells display uncontrolled rates of cellular proliferation and require an increased supply of precursor amino acids to support key biosynthetic pathways [1]. As a result, these cells have very efficient transport systems and can transport amino acid across their plasma membranes faster than normal cells can [1]. Glutamine provides nitrogen for a number of important precursors for macromolecule synthesis, including purines, pyrimidines, amino sugars, and some amino acids [2]. In addition, it is an important fuel for cancer cells. It has been shown for cancer cells that both cell growth rates and DNA and protein biosynthesis correlate directly with the concentration of glutamine in culture media [3].

Oxidative stress plays an important role in cancer growth and progression [4]. Glutathione, which serves as a major store of cellular reducing equivalents, is of critical importance to tumor cells, affecting their ability to withstand oxidative attack and their chemosensitivity and radiation sensitivity [5]. Tumor glutathione metabolism also regulates the metastatic behavior of malignant cells [6]. Glutathione is a tripeptide composed of glutamate, glycine, and cysteine. Glutamine metabolism can supply glutamate, a precursor for the synthesis of glutathione, by the hydrolysis of glutamine via the enzyme glutaminase [7]. Abcouwer reported that intracellular glutathione levels dropped rapidly when breast cancer cells were deprived of extracellular glutamine [8].

Neuroblastomas are biologically remarkable in that some regress spontaneously without chemotherapy, and spontaneous and induced maturation is seen with significant frequency [9]. Although many tumor markers have been investigated with respect to the biology of neuroblastomas, a definitive and consistent causal pattern for neuroblastomas' diverse behavior and variable biology is still unexplained.

Because of the potential role of glutamine as a mediator of tumor growth and glutathione production, we hypothesized that glutamine mediates cancer cell growth by regulating amino acid transport activity and glutathione levels, both when sufficient nutrients are present and when key nutrients such as glutamine are in limited supply. The purpose of this study was to examine the effects of glutamine deprivation on amino acid

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transport and glutathione levels in an SK-N-SH human neuroblastoma cell line. In addition, we studied the effects of glutamine deprivation on DNA and protein biosynthesis.

Materials and methods

Chemicals

Radiolabeled amino acids (^{3}H-L-glutamine, ^{3}H-L-glutamate, and ^{3}H-L-leucine) and ^{3}H-thymidine were obtained from Amersham (Arlington Heights, IL, USA). Cell culture media were from GIBCO/BRL (Gaithersburg, MD, USA). Amino acids and all biochemicals were purchased from Sigma Chemical (St. Louis, MO, USA), and fetal bovine serum was from JRH Biosciences (Lenexa, KS, USA). Tissue culture plates were obtained from Corning (New York, NY, USA). Neuroblastoma cell line, SK-N-SH, was provided by Dr. Tadao Ohno (RIKEN Cell Bank, Tsukuba, Japan).

Cell culture

Neuroblastoma cells were cultured at 37ºC under a humidified atmosphere of 5% CO_2/95% air. The cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 2 mM L-glutamine, 10% fetal bovine serum (FBS), 1,000 units/ml penicillin, and 1,000 units/ml streptomycin. The culture medium was changed every 3 days until cells were confluent, at which point the cells were used for experiments.

Cell growth measurement

The effect of glutamine concentration on cell growth was determined. Cells were seeded at a density of 1×10^5 cells/ml (1 ml/well) into 12-well tissue culture plates. After 24 h, the culture medium was removed and changed to glutamine-free DMEM supplemented with 10% FBS plus various concentrations of glutamine (2 mM, 400 µM, 200 µM, and 0 µM). After 2 days, cells were detached from the plate with trypsin and counted with a hemocytometer. Cell growth in 2 mM glutamine was chosen as the control.

Measurement of amino acid transport

Cells were seeded into 24-well tissue culture plates (0.5 ml/well) and maintained in DMEM supplemented with 2 mM glutamine and 10% FBS. After getting 100% cell confluence, the culture medium was removed and changed to DMEM supplemented with 10% FBS plus various concentrations of glutamine (2 mM, 400 µM, 200 µM, and 0 µM). Amino acid transport was measured at 24 h.

Amino acid transport was measured by the cluster tray method of Gazzola et al. [10]. Before the transport assays, the cells were rinsed twice with warm sodium-free Krebs-Ringer phosphate buffer (CholKRP, which was made by replacing the corresponding sodium salts with choline chloride and choline phosphate) to remove extracellular sodium and amino acids. The transport of radiolabeled amino acid (5 µCi ^{3}H-amino acid/ml) was performed for 1 min at 37ºC at 10 µmol/l unlabeled amino acid in both sodium Krebs-Ringer Phosphate (NaKRP) and CholKRP buffers. The transport reaction was terminated by discarding the uptake buffer and rinsing the cells three times with ice-cold buffer (2 ml/well/rinse). The wells containing the cells were allowed to dry and were solubilized in 200 µl of 0.2 N NaOH/0.2% sodium dodecyl sulfate solution. One hundred µl of the cell extract was neutralized with 10 µl 2 N HCL and subjected to scintillation spectrophotometry. The remaining 100 µl in each well was used for the protein assay by the bicinchoninic acid protein method [11].

The sodium-dependent transport values were obtained by subtracting the transport values in CholKRP from those in NaKRP. Saturable sodium-independent transport values were determined in CholKRP by subtracting the values in the presence of excess (10 mM) unlabeled amino acid from those in its absence.

Measurement of DNA and protein synthesis

To determine DNA and protein synthesis, we measured the incorporation of ^{3}H-thymidine and ^{3}H-leucine, respectively, into acid-insoluble material. Cells were seeded in 24-well cluster trays (0.5 ml/well). After getting 100% cell confluence, the medium was removed and replaced with DMEM plus 10% FBS and various concentrations of glutamine (2 mM, 400 µM, 200 µM, and 0 µM). After 24 h, ^{3}H-thymidine and ^{3}H-leucine (1 µCi/ml) were added to the culture medium, and cells were incubated for 2.5 h at 37ºC. The assay was terminated after 2.5 h, when the cells were washed twice with phosphate-buffered saline (PBS) and fixed by washing three times with ice-cold 10% trichloroacetic acid. Thereafter, cells were rinsed twice with 70% and 95% ethanol, respectively. They were allowed to dry and solubilized in 200 µl of 0.2 N NaOH/0.2% sodium dodecyl sulphate solution. Radioactivity and protein content were measured by the same procedures described for amino acid transport measurements.

Measurement of glutathione

To determine glutathione levels, equal number of cells was seeded in each 100-mm dishes. After getting 100% cell confluence, the medium was removed and replaced with DMEM plus 10% FBS and various concentrations of glutamine (2 mM, 400 µM, 200 µM, and 0 µM). After 24 h, cells were rinsed twice with PBS and