(0.20 ± 0.06), and the third Han from Xi'an and the Sali, a branch of the Yi ethnic group (0.15 ± 0.06). Ryukyuans and Ainu behave as outliers with significant differences with all the samples. Population groups resulting from the F_{ST} and CLUSTER analysis are defined in Table 3. Although mainland Japanese from Aichi were significantly different from other mainland Japanese because of their high frequency of haplogroup B, they were merged with them as JPN for comparisons with other areas. Control of the conglomerate number expected in CLUSTER analysis allows for a hierarchical grouping of populations. With two conglomerates, the first distinguished isolate was the aboriginal Sakai from Thailand (Fucharoen et al. 2001). This group was unique among other Thai people owing to its lack of lineages with the 9-bp deletion that characterizes haplogroup B, and to the high frequency of the authors' C6 cluster (included in our D4a). The lack of any representative of macrohaplogroup N in a population anthropologically considered one of the oldest groups in Thailand, if not caused by genetic drift, is compatible with the hypothesis that derivatives of macrohaplogroup N had, in southern Asia, a different route from macrohaplogroup M (Maca-Meyer et al. 2001). Also striking is the presence in Sakai of an unequivocal representative (16223–16274–16278–16294–16309) of the sub-Saharan African L2a haplogroup (Torroni et al. 2001), which again is compatible with the physical characteristics of this Negrito group. Although the suggestion that the first spreading out of Africa of modern humans could have carried some L2 lineages in addition to the L3 ancestors (Watson et al. 1997) is a tempting explanation, a recent admixture is more in consonance with the phylogenetic proximity of this lineage to the present African ones. The next outsiders were the majority of the Siberian isolates, which could not be pooled because of big differences in the frequency of distinctive haplogroups (Table 2). This considerable differentiation was already emphasized (Schurr et al. 1999), with strong genetic drift being its most probable cause. Subsequent isolates belong to some Chinese minorities such as those of Lisu and Nu, Lahu, and Taiwanese aborigines. Unexpectedly, other Chinese minorities (Bai, Sali, and Tu) were left in Han Chinese northern clusters. The Bai belong to the Sino-Tibetan Tibeto-Burman ethnic linguistic group and have been strongly influenced by Han. The Sali are a minority within the Yi ethnic group whose most probable ancestors were the Qiang from northwest China. Finally, the Tu, although belonging to the Mongolian branch of the Altaic Family, show their main genetic affinities to the Han from Xi'an ($P = 0.95$), Xinjiang ($P = 0.89$), and Shanghai ($P = 0.79$), all of them clustered in the Ch2 group. On the other hand, Thais, Vietnamese, and Cambodians joined with southern Chinese. As already observed (Chunjie et al. 2000; Yao et al. 2002a), the Han Chinese do not comprise a homogeneous group. With the exception of cluster Ch4, that includes samples from Hubei and Guandong (Table 3), they appear geographically differentiated. The two central Asian groups detected mainly differ in their frequencies for A1b, Z, and G2a. With less than 14 conglomerates, the Japanese, including Ainu and Ryukyuans, were part of a big group formed by Korean, Buryat, Tibetans, and northern Chinese. Ainu was the first differentiated Japanese sample. Ryukyuans separated later, when mainland Japanese and Koreans still comprised a single group. The lack of homogeneity between Ainu and Ryukyuans was pointed out by Horai et al. (1996), who questioned that they shared a recent common ancestor. The main differences between them were attributed to two dominant clusters (C1 and C16, corresponding to our Y and M5/D4a/G1, respectively) present in Ainu but absent in Ryukuyans, and two Ryukyuan dominant clusters (C3 and C13, belonging to our R and M, respectively) absent in Ainu. In addition, applying the present haplogroup nomenclature to the same data, the high frequency of M7a1 and D4a1/D4b in Ryukyuans, but their absence in Ainu, stands out. The MDS plot (Fig. 3A), based on F_{ST} haplogroup frequency distances between final groups (data not shown), only partially reflects the sequential process described above, as only Sakai and Siberians are well differentiated from the rest. On the contrary, relationships obtained from haplotype matches (Fig. 3B) show populations highly structured by geography with the only exceptions being the Ainu and Tuvinian isolates.

The Peopling of Japan

To further know the relative affinities of the Japanese between themselves and with the different Asian groups formed, the data obtained from the global approaches based on haplogroup frequency distances and on sequence match identities are presented in Table 4. Both values are moderately correlated in the comparisons involving the mainland Japanese ($r = -0.479$; two-tail probability 0.012) but not at all in those involving aborigine Ryukyuans ($r = -0.310$; two-tail probability 0.115) and Ainu ($r = 0.087$; two-tail probability 0.667). This result can be explained by assuming that these aboriginal people have suffered important genetic drift effects with substantial changes in haplogroup frequencies and lineage losses or, less probably, that these populations have been isolated long enough to have accumulated new variation. Results based on haplogroup frequencies by far relate mainland Japanese to Koreans followed by northern Chinese. Ryukyuans present the smallest distances to Buryats from South Siberia, followed in short by southern Chinese. In turn, the Ainu have their closest affinities with mainland Japanese, Koreans, and northern Chinese. As regards sequence matches, mainland Japanese also joins first to Koreans and second to Buryats. Aborigine Ryukyuans are closest to Buryats and then to Koreans. Finally, Ainu show comparatively less shared sequences, their greater affinities being toward Chukchi and Koryaks of Kamchatka. This global picture is congruent with an important influence on mainland Japanese from northern Asian populations through Korea, that the Ryukyuans had a dual northern and southern Asian background previous to the new northern influences acquired by admixture with mainland Japanese, and that the Ainu represent the most isolated group in Japan in spite of the genetic input received from Kamchatka. Also noticeable is the great distance and low identity values obtained for the Ainu–Ryukyuan pair compared with those obtained in their respective comparison to mainland Japanese, which is another hint of its notable maternal isolation.

The distance and identity statistics used above are based on frequencies of haplogroups and haplotypes, respectively; however, frequencies are more affected by genetic drift than the number of different haplotypes present in a population. To measure the relative affinities of Japanese populations between them and to Continental Asia in a frequency-independent way, we chose a haplotype-sharing approach calculating the relative contribution of lineages shared with other areas to the number of different haplotypes present in each Japanese population. In these comparisons all other Asians were merged. Table 5 shows the results of this analysis. Note that despite the difference in sample size the haplotype frequency in mainland Japanese and Ainu is ~50%, whereas in Ryukyuans it is 84%; which means that, if there was not a bias in the sampling process, in spite of its small size, the Ainu sample seems to be representative of that population. However, it would be desirable to enlarge that of the Ryukyuans (Helgason et al. 2000). Haplotypes present only in a given population account for 13% in Ainu but ~50% in mainland Japanese (60%) and Ryukyuans (45%). This finding once more points to the existence of important drift effects in Ainu. Mainland Japanese exclusively share with Ryukyuans and Ainu only 3% and 2%, respectively, of its lineages, which could reach 6% and 3% if those

Table 3. Asian Populations Used in This Study

Population	Locality	Ethnic group	Group	Sample	HVRI	HVRII	Other[a]	References
Japan	Tokyo	Japanese	JPN	373	16024–16569	1–648	649–16023	This work
Japan	Nagoya	Japanese	JPN	299	16024–16569	1–648	649–16023	This work
	Japan	Japanese	JPN	20	1600–16413	—		Bamshad et al. 2001
				19	—	71–270		Jorde et al. 1995
Japan	Tokyo	Japanese	JPN	162	16051–16365	73–340		Imaizumi et al. 2002
Japan	Tokyo	Japanese	JPN	150	16030–16481			Nishimake et al. 1999
Japan	Tokyo	Japanese	JPN	13	16024–16569	1–648	RFLPs	Abe et al. 1998
Japan	Miyazaki	Japanese	JPN	100	15998–16400	30–407		Seo et al. 1998
Japan	Tottori	Japanese	JPN	89	16026–16396			Oota et al. 2002
Japan	Shizuoka	Japanese	JPN	62	16129–16569	1–41		Horai et al. 1996
Japan	Aichi	Japanese	JPN	50	16040–16375	20–430		Koyama et al. 2002
Japan	Okinawa	Ryukyuan	RYU	50	16129–16569	1–41		Horai et al. 1996
Japan	Hokkaido	Ainu	AIN	51	16129–16569	1–41		Horai et al. 1996
Korea		Korean	KOR	306	16020–16400	1–70		Lee et al. 1997
Korea		Korean	KOR	4	16024–16370			Torroni et al. 1993a,b
Korea		Korean	KOR	60	16024–16365	73–340		Pfeiffer et al. 1998
Korea		Korean	KOR	2	16000–16413	—		Bamshad et al. 2001
Korea					—	71–270		Jorde et al. 1995
Korea		Korean	KOR	64	16129–16569	1–41		Horai et al. 1996
Korea		Korean	KOR	3	16128–16408			Horai and Hayasaka 1990
Korea		Korean	KOR	98	16075–16362	73–315	14747–15887	Lee et al. 2002
China	Liaoning	Han	Ch1	51	16001–16497	30–47	10171–10659 and RFLPs	Yao et al. 2002a
China	Shandong	Han	Ch1	50	16001–16497	30–47	10171–10659 and RFLPs	Yao et al. 2002a
China	Yunnan	Bai	Ch1	31	16001–16495			Yao et al. 2002b
China	Changsha	Han	Ch1	82	16026–16396			Oota et al. 2002
China	Xinjiang	Han	Ch2	47	16001–16497	30–47	10171–10659 and RFLPs	Yao et al. 2002a
China	Yunnan	Sali	Ch2	31	16001–16495			Yao et al. 2002b
China	Qinghai	Tu	Ch2	35	16001–16495			Yao et al. 2002b
China	Xi'an	Han	Ch2	84	16026–16396			Oota et al. 2002
China	Shanghai	Han	Ch2	120	13030–16481			Nishimake et al. 1999
Mongolia		Mongolian	Ch2	103	16020–16400		RFLPs	Kolman et al. 1996
Mongolia		Mongolian	Ch2	15	16001–16495			Yao et al. 2002b
China	Yunnan	Lahu	Ch3	32	16048–16569	1–49		Qian et al. 2001
China	Hubei	Han	Ch4	42	16001–16497	30–47	10171–10659 and RFLPs	Yao et al. 2002a
China	Guangdong	Han	Ch4	30	16001–16497	30–47	10171–10659 and RFLPs	Yao et al. 2002a
China	Yunnan	Han	Ch5	43	16001–16497	30–47	10171–10659 and RFLPs	Yao et al. 2002a
China	Taiwan		Ch5	6	16024–16370			Torroni et al. 1993a,b
China	Taiwan		Ch5	3	15999–16413			Bamshad et al. 2001
China	Taiwan		Ch5	9	16065–16375			Sykes et al. 1995
China	Taiwan		Ch5	66	16129–16569	1–41		Horai et al. 1996
China	Taiwan	Han	Ch5	155	15997–16569	1–407		Tsai et al. 2001
China	Yunnan	Dai	Ch5	21	16048–16569	1–49		Qian et al. 2001
China	Yunnan	Wa	Ch5	22	16048–16569	1–49		Qian et al. 2001
China	Yunnan	Dai	Ch5	38	16001–16495			Yao et al. 2002b
China	Guangxi	Zhuang	Ch5	83	16001–16495			Yao et al. 2002b
China	South China	Han	Ch5	28	16024–16399			Betty et al. 1996
Thailand			Ch5	32	16001–16495			Yao et al. 2002b
Thailand		See ref.	Ch5	121	16048–16569	1–41		Fucharoen et al. 2001
Thailand	See ref.	Native	Ch5	74	16048–16569	1–41		Fucharoen et al. 2001
Vietnam			Ch5	35	16026–16396			Oota et al. 2002
Vietnam			Ch5	9	15999–16413	—		Bamshad et al. 2001
					—	71–270		Jorde et al. 1995
Cambodia			Ch5	12	15999–16413	—		Bamshad et al. 2001
					—	71–270		Jorde et al. 1995
China	Yunnan	Lisu	Ch6	37	16001–16495			Yao et al. 2002b
China	Yunnan	Nu	Ch6	30	16001–16495			Yao et al. 2002b
China	Taiwan	Native	TWA	28	15997–16400	30–407		Melton et al. 1998
China	Taiwan	Native	TWA	180	16048–16569	1–41		Tajima et al. 2003
Central Asia		Uygur	CA1	46	16001–16495			Yao et al. 2000a
Kazagstan		Kazakh	CA1	55	15997–16400			Comas et al. 1998
Kirgizistan	Talas	Kirghiz	CA1	48	15997–16400			Comas et al. 1998
Kazagstan		Uygur	CA1	55	15997–16400			Comas et al. 1998
Central Asia		Kazak	CA2	30	16001–16495			Yao et al. 2000a
Kirgizistan	Sary-Tash	Kirghiz	CA2	46	15997–16400			Comas et al. 1998
Siberia	See ref.	Altai	CA2	17	16024–16383			Shields et al. 1993
Tibet		Tibetan	TIB	1	16024–16370			Torroni et al. 1993a,b
Tibet		Tibetan	TIB	40	16001–16495			Yao et al. 2000b
Tibet		Tibetan	TIB	24	16048–16569	1–41		Qian et al. 2001
Russia	East Ural	Mansi	MAN	98	16039–16519	64–295	RFLPs	Derbeneva et al. 2002a

(continued)

Table 3. *Continued*

Population	Locality	Ethnic group	Group	Sample	HVRI	HVRII	Other[a]	References
Siberia		Finno-Ugrian	FIU	38	13021–16505			Voevoda Accession nos. AF214068–AF214105
South Siberia		Tuvinian	TUV	36	16000–16400		RFLPs	Derenko et al. 2000
South Siberia		Buryat	BUR	40	16000–16400		RFLPs	Derenko et al. 2000
Siberia		Chukchi	CHU	60	16001–16405			Voevoda et al. 1994
Siberia	Aluitor	Koryak	ALU	56	16000–16525			Schurr et al. 1999
Siberia	Karagin	Koryak	KAM	37	16000–16525			Schurr et al. 1999
Siberia	Palan	Koryak	KAM	54	16000–16525			Schurr et al. 1999
Siberia	Kovran	Itel men	ITE	46	16000–16525			Schurr et al. 1999
Philippine			FIL	32	16065–16375			Sykes et al. 1995
Thailand	Trang	Sakai	SAK	20	16048–16569	1–41		Fucharoen et al. 2001
Malaysia			IND	6	15999–16413	—		Bamshad et al. 2001
					—	71–270		Jorde et al. 1995
Indonesia			IND	34	16024–16400	31–407		Redd and Stoneking 1999
Borneo		Sabah	SAB	34	16065–16375			Sykes et al. 1995

[a]RFLPs and additional sequences.

also shared with Continental Asian populations are added. In comparison they shared 21% of its lineages with other Asians. On the contrary, Ryukyuans and Ainu share about 50% of their lineages with mainland Japanese and only 10% and 21%, respectively, with Continental populations, which may reflect other independent Asian influences on Japan. With respect to those lineages exclusively shared by Japanese and Continental Asian populations, it is worth mentioning that, again, Korea is the main contributor, participating in ~50% of the haplotype sharing with mainland Japanese (55%), as much as with Ryukyuans (50%) and Ainu (50%). However, differences exist in the provenance of the rest of the shared lineages. Whereas in Ainu (northern China and Siberia) and in Ryukyuans (northern China and central Asia) they are from northern areas, the second region contributing to mainland Japanese is southern China (17.5%), followed, at the same level (12.5%), by northern China and central Asia. In addition, there exists a minor percentage of exclusive sharing with Indonesia (2.5%). On the other hand, all the matches with Siberia and Tibet are also shared with other populations. From these results, it can be deduced that the ancient Japanese inhabitants came from northern Asia and that southern areas affected the Japanese by later immigration. Nevertheless, it must be borne in mind that older influences could be undetectable by lineage sharing. With respect to the haplogroup affiliation of those lineages that Ainu and Ryukyuans exclusively shared with no Japanese samples, new differences appear between them. Ainu share derived lineages of haplogroups A, G, M9, and D5, all of them compatible with a rather recent Siberian influence. In contrast, those shared by Ryukyuans are basical M lineages, more congruent with an older radiation from southern China. These dual influences are also detected when the haplogroup affiliation of the Ainu and Ryukyuan unique lineages is studied. First, the percentage of lineages belonging to macrohaplogroup N is larger in Ainu (50%) than in Ryukyuans (15%) and from a different provenance, as those in Ainu are from haplogroups N, N9b, and Y, whereas those of Ryukyuans belong to the southern haplogroups F and B. The remaining 50% of the Ainu lineages equitably belong to different M haplogroups (M, M7c, G1, and D5a), but in Ryukyuans the remainder are mainly concentrated in M7a (41%) and M7b2 (18%), two groups that have their greatest Asian diversities precisely in Ryukyuans. Although an indigenous focus of radiation cannot be discarded, it is more conservative to suppose that the most probable origin of these lineages is again southern China. Thus, Ainu and Ryukyuans are not only largely isolated populations, but they most probably had different maternal origins.

Although no matches are involved, the geographic distribution of haplogroup frequency and diversities for some groups present in Japan and in other distinct Asian areas are also relevant to trace these older connections. For instance, haplogroups M9, M10, M12, D4b, and F1c have correlated geographic frequencies with a peak in an area that comprises Tibet (Table 2). Curiously, one of these haplogroups (M12) is today absent in China but present in Korea and Japan.

DISCUSSION

Although the recent out-of-Africa origin for all modern humans (Cann et al. 1987) is being widely supported (Takahata et al.

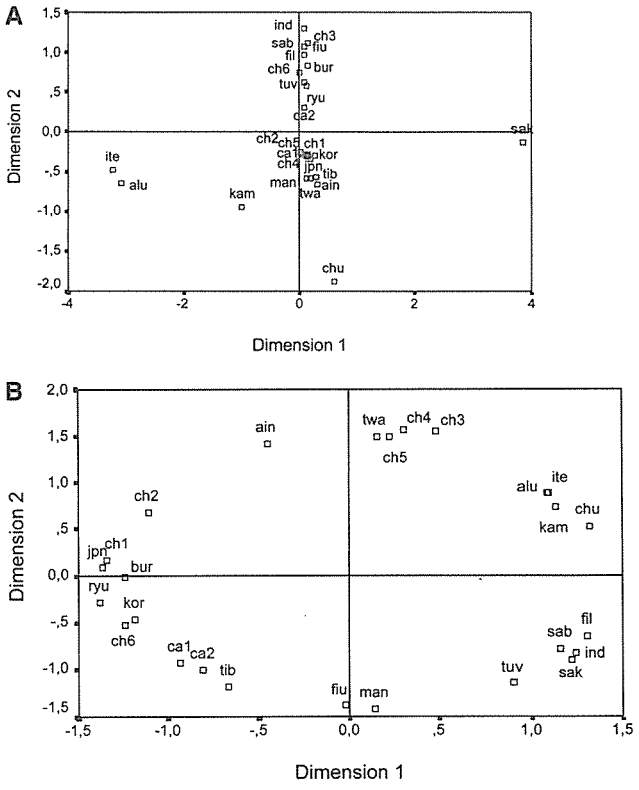

Figure 3 MDS plots based on (*A*) F_{ST} and (*B*) D match distances. Population groups are as detailed in Table 3.

Table 4. Frequency-Based F_{ST} and Sequence Match Identities (in Percentage) Between Japanese Samples and With Other Asian Populations

	JPN		RYU		AIN	
	F_{ST}	Matches	F_{ST}	Matches	F_{ST}	Matches
RYU	0.04	0.41				
AIN	0.04	0.33	0.05	0.04		
KOR	0.00	1.10	0.04	0.57	0.04	0.25
CH1	0.01	0.59	0.04	0.11	0.04	0.18
CH2	0.01	0.51	0.05	0.19	0.05	0.21
CH3	0.07	0.01	0.10	0.00	0.08	0.00
CH4	0.03	0.06	0.03	0.00	0.05	0.03
CH5	0.03	0.16	0.03	0.09	0.05	0.08
CH6	0.04	0.01	0.08	0.00	0.08	0.09
TWA	0.04	0.23	0.07	0.08	0.08	0.04
TIB	0.04	0.36	0.04	0.18	0.08	0.06
CA1	0.02	0.58	0.04	0.25	0.05	0.16
CA2	0.04	0.73	0.07	0.20	0.08	0.19
ITE	0.29	0.00	0.39	0.00	0.40	0.26
FIU	0.06	0.50	0.08	0.32	0.10	0.10
MAN	0.06	0.24	0.06	0.24	0.08	0.04
ALU	0.29	0.01	0.39	0.00	0.39	0.46
KAM	0.14	0.01	0.16	0.00	0.15	0.45
CHU	0.17	0.01	0.21	0.00	0.22	0.00
TUV	0.03	0.09	0.07	0.17	0.07	0.05
BUR	0.03	0.97	0.02	2.75	0.07	0.15
FIL	0.03	0.11	0.05	0.13	0.06	0.00
IND	0.09	0.04	0.09	0.00	0.11	0.00
SAK	0.29	0.00	0.44	0.00	0.43	0.00
SAB	0.06	0.09	0.05	0.29	0.08	0.12

2001), the most probable time and routes chosen by these earliest migrants to reach eastern Asia is an open issue. In the following discussion we weigh the different alternatives proposed in light of the phylogenetic tree obtained from complete mtDNA sequences. One of the first questions raised was whether there was more than one out-of-Africa dispersion. All the mtDNA lineages detected in Old World populations belong to one of two M and N macrohaplogroups with only secondary representatives in Africa. The proposed radiation ages for both, 30,000 to 58,000 years ago and 43,000 to 53,000 years ago, respectively (Maca-Meyer et al. 2001), give a temporal frame compatible with only one main dispersion or two successive dispersions, in which case the M precursor is the most probable candidate for the older exit. Even if the one dispersion option is chosen, more than one geographical route to eastern Asia is possible. In fact, a northern Continental route through the Near East and western-central Asia and a southern coastal route through the Arabian and Indian peninsulas have been proposed (Cavalli-Sforza et al. 1994; Kivisild et al. 1999). The geographical distribution of these two macrohaplogroups, with lack of ancient M representatives and the presence of deep N lineages in western Asia, and the abundance of basal M lineages in India and southwestern Asia and concomitant lack of equivalent-age N clades, gave rise to the hypothesis that N represents the main footprint of the northern Continental expansion, whereas M is the equivalent footprint for the southern coastal expansion. The presence of N and M lineages in alternative areas has been explained to have been the result of secondary migrations (Maca-Meyer et al. 2001). However, another plausible explanation is that both M and N reached southern Asia at the same time, quickly expanding to Papua New Guinea (PNG) during maximal glacial ages when the permafrost boundary precluded a northern human occupation. During postglacial ages, subsequent migrations northward carried derivatives of both macrohaplogroups to northern Asia (Forster et al. 2001). Nevertheless, under this second hypothesis, the presence of basal N clusters should be expected in India, southern Asia, and PNG; but this is not the case. All N representatives in India belong to R, a clade derived from N by the loss of 16223 and 12705 mutations (Fig. 2). In addition, the bulk of these Indian lineages belong to western Caucasian haplogroups that, most probably, reached India as the result of secondary immigrations, as has already been proposed (Kivisild et al. 1999; Bamshad et al. 2001). Similarly, the N representatives in southern Asia belong to haplogroups F and B, two sister clades also derived from R (Fig. 2). Furthermore, when totally sequenced PNG N lineages (Ingman et al. 2000; Ingman and Gyllensten 2003) are added to the N phylogenetic tree (data not shown), they form three monophyletic clades that have their roots in the derived R trunk. On the contrary, the geographically northern Asian clades A, N9a, N9b, and Y (Fig. 2) and the western Eurasian clades W, N1b, I, and X all split from the basal N root (Maca-Meyer et al. 2001), although A, N9a, N9b, and Y radiations were delayed congruent with subsequent northern Asian expansions. Therefore, at present, mtDNA data are compatible with the supposition that the northern route, harboring mainly N precursors, met climatic difficulties and when they finally reached Southeast Asia, the M representatives, brought by the southern route, had already colonized the area. This southern expansion of N derivatives has, as a lower temporal boundary, the coalescence ages of F, B, and PNG R haplogroups being ~46,000 ± 10,000 years ago. However, when recently published (Ingman et al. 2000; Ingman and Gyllensten 2003) Australian N lineages are taken into account, it seems evident that the real situation could be far more complex than the one migration–one lineage hypothesis. Australian N lineages directly sprout from the basal trunk (data not shown). They most probably differentiated in that continent, supporting the idea that ancestral N lineages reached Australia but not PNG, although the undemonstrable possibility of lineage extinctions and subsequent recolonization events in PNG can be an argument. Both hypotheses have difficulties to explain the presence of ancient N lineages in Australia. If the two, M and N lineages, were brought with the southern coastal dispersion, the lack of primitive N in India, southern Asia, and PNG has to be explained by the subsequent loss of all N lineages carried to Australia; if the northern Continental route of N is favored, the loss of N representatives in all populations formed in route to Australia has also to be explained. Recently, an N lineage has been detected in Chenchus, a southern Indian tribal group (Kivisild et al. 2003). From the information published, it can be deduced that this lineage only shares mutation 1719 with the western Eurasian Nb1/I and X clades. More extensive studies of populations in southern India

Table 5. Distribution of Unique and Shared Haplotypes in Japanese Populations

	Japanese populations		
	JPN	RYU	AIN
Sample	1318	50	51
Haplotypes	626	42	24
Haplotype frequency	0.48	0.84	0.47
Singleton + Unique	377 (0.60)	19 (0.45)	3 (0.13)
Shared	249 (0.40)	23 (0.55)	21 (0.87)
JPN	137 (0.22)	20 (0.48)	13 (0.54)
RYU	20 (0.03)	1 (0.02)	1 (0.04)
AIN	13 (0.02)	1 (0.02)	5 (0.21)
Other[a]	130 (0.21)	4 (0.10)	5 (0.21)

[a]Other Asians.

and southern and central Asia would add empirical support to any of these theories.

Concerning macrohaplogroup M, it has already been commented that the star radiation of all the main Indian and southeast Asian M clades strongly suggests that this wide geographic colonization could have happened in a relatively short time (Maca-Meyer et al. 2001). This star radiation includes the Australian and PNG M complete sequences recently published (Ingman et al. 2000; Ingman and Gyllensten 2003). However, for those clades and subclades with later northward expansions, long radiation delays are observed. For instance, whereas M7 and M8 have coalescence ages ~35,000 to 45,000 years ago, other groups such as G, D4, M7a, or M7c have coalescence ages ~15,000 to 30,000 years ago, more in frame with those calculated for A, Y, and N9 derivates, which, although belonging to macrohaplogroup N, share with them a central–northern Asian geographic distribution (see Supplemental material). It seems that the simultaneous lineage bursts ~60,000 to 70,000 years ago from Africa (Maca-Meyer et al. 2001), ~30,000 to 55,000 years ago for macrohaplogroups M and N, and ~15,000 to 30,000 years ago for clusters with prominent central–northern Asian radiations were related to main climatic changes. The role of selection in these expansions is an open question (Elson et al. 2004; Ruiz-Pesini et al. 2004).

The application of global pairwise-distance and detailed phylogeographic methods to the peopling of Japan shows that both approaches have different grasps but together demonstrate that the actual Japanese population is the result of a complex demographic history, from which the different theories proposed to explain it only emphasize partial aspects. Global distances and detailed haplotype comparisons confirm that Ainu and Ryukyuans are heterogeneous populations (Horai et al. 1996) and that both are well differentiated from the mainland Japanese. In spite of this, they have common peculiarities such as having the highest frequencies in Asia for M7a, M7b2, and N9b, shared with mainland Japanese. Furthermore, for both, their closest relatives are northern populations. At first sight, these results are against a supposed southern origin for the Paleolithic Japanese, favoring the replacement theory or even that the Paleolithic inhabitants of Japan came from northeastern Asia (Nei 1995). Although based on a single locus, our results are strikingly coincident with the previously proposed northern origin and influences received by the Japanese. In an early study using serum gammaglobulin polymorphisms, it was concluded that the homeland of all Japanese could have been in the Lake Baikal area in Siberia (Matsumoto 1988), which agrees with the close proximity found here between Buryats and Ryukyuans or mainland Japanese. More recently, classical markers (Omoto and Saitou 1997) and mtDNA (Horai et al. 1996) studies demonstrated that the Japanese are most closely related to the Koreans, which is also true in our global analysis. It can be added that a substantial part of this common maternal pool has recent roots, as Korea specifically shares with Ainu, mainland Japanese, and Ryukyuans 10%, 7%, and 5%, respectively, of their haplotypes. This particular affinity is increased with the existence of derived lineages only detected (A1a, B4c1, B4f) or mainly detected (N9b, B4a1, B4b1, G1a, M7b2, M12) in Japanese and Koreans. This Korean influence has been attributed to the archeologically well-documented Continental immigration to Japan during the Yayoi period (Horai et al. 1996). However, specific haplotype matches with other areas increases the geographic range of these recent influences. Thus, mainland Japanese share part of their haplotypes exclusively with South China (2.5%), North China (1.5%), Central Asia (1.5%), and Indonesia (0.3%); and, also, Ryukyuans have specific affinities with North China (2.4%) and Central Asia (2.4%). The recent Siberian input on the Ainu has also been stressed (Schurr et al. 1999). At least, another independent migratory wave from central Asia also affected mainland Japanese. It was first detected by the peculiar distribution of the Y-chromosome marker YAP+, and seems to have originated in an area including Tibet (Su et al. 2000). Haplogroup M12 is its mitochondrial counterpart. As with the Y-chromosome marker, its punctual presence in Tibet and eastern Asia might be explained as the result of subsequent migrations in the Continent that erased the route followed by the people harboring these markers. In addition, there are clues, at least in Ryukyuans, that a substantial part of their maternal pool had an ancient southern Asian provenance. This fraction is represented by the M, M7a, and M7a1 basic lineages (31%), which the Ryukyuans do not share with northern populations. This southern signal is, in part, congruent with the southern Asian origin for the Paleolithic Japanese proposed by the dual structure model (Hanihara 1991). Furthermore, the fact that the highest diversities for M7a, M7a1, and M7b2 have been found in Ryukyuans and for N9b and B5b2 in Japan raises the possibility that this area was within a focus of migratory radiations to northern and southern isles and even to the mainland from Paleolithic to recent times. The significant latitudinal clines detected in Japan for some genetic markers (Orito et al. 2001; Takeshita et al. 2001) could also be explained as the result of southern and northern influences on Japanese. Finally, some mtDNA results obtained from ancient Jomon remains (Horai et al. 1991; Shinoda and Kanai 1999; K.-I. Shinoda, unpubl.) are congruent with a genetically diverse background for the Paleolithic Japanese population (Horai et al. 1996). A tentative comparison of Jomon with present-day Japanese populations based on shared lineages (data not shown) significantly relates Jomon first to the indigenous Ainu and then to Ryukyuans and last to mainland Japanese. In summary, Japan could have received several northern and southern Asian maternal inputs since Paleolithic times, with notable northern Asian immigrations through Korea in the late Neolithic and more specific gene flows from western Asia, Siberia, and southern islands.

METHODS

Samples

Complete mtDNA sequences were obtained from a total of 672 unrelated Japanese including 373 from Tokyo and 299 from the Nagoya area. All subjects gave their written consent to participate in this study, which was approved by the Ethical Committees of the Gifu International Institute of Biotechnology and collaborative institutions. The sources of 11 additional complete sequences used to build the final phylogenetic trees are in Table 1. For the analysis of the peopling of Japan, we used a total of 1438 Japanese and 3275 central and eastern Asian HVI sequences, as detailed in Table 3.

Isolation and Amplification of DNA

Total DNA was extracted from the blood with either Dr. Gen TLE (Takara) or MagExtractor System MFX-2000 (Toyobo). The entire mitochondrial genome was amplified as six fragments (~3000–3400 bp) by the first PCR and 60 overlapping segments (~600–1000 bp) by the second PCR. The primer pairs and their nucleotide sequences were described previously (Tanaka et al. 1996). The conditions for the first and second PCR were the same: an initial denaturation step for 5 min at 94°C, followed by 40 cycles of denaturation for 15 sec at 94°C, annealing for 15 sec at 60°C, and extension for 3 min at 72°C, with a final extension for 10 min at 72°C. The amplified fragments were analyzed by electrophoresis on a 1% agarose gel and visualized by staining with ethidium bromide. These second PCR products were purified by use of the MultiScreen-PCR Plates (Millipore). The quality of DNA templates was examined by electrophoresis on a 1.2% agarose gel after staining with ethidium bromide by use of a Ready-To-Run Separation Unit (Amersham Pharmacia Biotech).

Sequence Analysis of Mitochondrial DNA

Sequence reactions were carried out with a BigDye terminator cycle sequencing FS ready reaction kit (Applied Biosystems). After excess dye terminators had been removed with MultiScreen-HV plates (Millipore) packed with Sephadex G50 superfine (Pharmacia), the purified DNA samples were precipitated with ethanol, dried, and suspended in the template suppression reagent (TSR) or formamide from Applied Biosystems. The dissolved DNA samples were heated for 2 min at 95°C for denaturation, then immediately cooled on ice. Sequences were analyzed with automated DNA sequencers 377 and 310 by use of Sequencing Analysis Program version 4.1 (Applied Biosystems). A computer program, Sequencher version 4.1 (Gene Codes Co.), was used to indicate possible single nucleotide polymorphism (SNP) loci. For verification, visual inspection of each candidate SNP was carried out. At least two overlapping DNA templates amplified with different primer pairs were used for identification of each SNP. Mitochondrial SNPs (mtSNPs) were identified by comparison with the revised Cambridge sequence (rCRS) reported by Andrews et al. (1999).

Phylogenetic Analysis of Complete Coding-Region mtDNA Sequences

In this present study, nucleotide positions were numbered as in the Cambridge Reference Sequence (CRS; Anderson et al. 1981), nucleotide substitutions were expressed as differences from the revised CRS (Andrews et al. 1999), transitions were denoted only by their nucleotide positions, and transversions were designated by their nucleotide positions followed by the changed base. A total of 942 complete coding-region mtDNA sequences, including our 672 Japanese; one additional Japanese (GenBank accession no. AB055387); 53 worldwide sequences (Ingman et al. 2000); 42 worldwide sequences (Maca-Meyer et al. 2001); two Finnish sequences having Asian relatives (Finnilä et al. 2001); 17 Asian sequences without concrete geographic assignation (Herrnstadt et al. 2002); 37 sequences from the Bering area (Derbeneva et al. 2002b); 70 Asian, New Guinean, and Australian sequences (Ingman and Gyllensten 2003); and 48 Chinese sequences (Kong et al. 2003) were aligned with the rCRS by CLUSTAL V software, and the coding region was used to construct a phylogenetic network (Bandelt et al. 1999) rooted with a chimpanzee sequence (GenBank accession no. D38113) as implemented in the Network 3.1 program (Fluxus Engineering; http://www.fluxus-engineering.com). The noncoding positions were added by hand using molecular weighted parsimony criteria (Bandelt et al. 2000). The phylogenetic relationships obtained were also confirmed by means of a neighbor-joining tree (1000× bootstrapped; Saitou and Nei 1987), built using MEGA2 (Kumar et al. 2001). From this network (see Supplemental material) we chose 102 Japanese and nine Asiatic sequences that represented the main clusters and subclusters within the two macrohaplogroups M and N that colonized Asia. To define these groups we followed the most generalized cladistic nomenclature actually used to classify mtDNA lineages (Richards et al. 1998). For the haplogroups previously detected, we maintained the same notation as their authors proposed (Richards et al. 2000; Bamshad et al. 2001; Kivisild et al. 2002; Yao et al. 2002a; Kong et al. 2003). Those haplogroups introduced here for the first time were named according to their phylogenetic range deduced from the tree of complete sequences.

Haplogroup Assorting of Published Partial mtDNA Sequences

The unambiguously classified complete mtDNA sequences were used as an initial pool that was hierarchically enlarged by the successive addition of those published partial mtDNA sequences with the largest coding information, ending with those for which information on only control-region sequences for both mtDNA hypervariable segments or just one (HVS-I and/or HVS-II) was available, always following sequence matches or, as default, sequence-relatedness criteria. Some of those partial sequences that could be assigned to more than one haplogroup were tentatively assorted in the most probable one deduced from their geographic origin and the relative haplogroup distribution.

Pooling Small Size Samples and Rare Clades

To avoid small sample sizes and rare alleles in population comparisons, samples with <20 individuals were pooled with others from the same geographic and ethnic group. Within populations, individuals belonging to rare clades were pooled with those classified in the nearest branch. Pairwise sample distances were calculated as linearized F_{ST} distances as implemented in the ARLEQUIN program (Schneider et al. 2000), taking mtDNA as one locus with as many alleles as the different subhaplogroups considered.

Quantitative Affinities of Japanese Samples

Relative affinities of Japanese samples to the other Asiatic populations were assessed by linearized F_{ST} distances, using subhaplogroup frequencies, and haplotype matches' distances (D) estimated simply as $D = 1 - \sum(x_i y_i)$, x_i and y_i being the frequency of haplotype i in the two compared populations. To be statistically robust, these analyses require large sample sizes, thus further pooling was necessary. Previous studies in the area prevented us from pooling populations by geographic proximity (Schurr et al. 1999) and/or ethno-linguistic relationship (Comas et al. 1998; Chunjie et al. 2000; Yao et al. 2002a). For this reason, a genetic affinity criterion was chosen. Two approaches were used. In the first, all samples with no significant F_{ST} distances between them and with a similar behavior to the rest of the samples studied, were grouped. In the second, pooling was carried out by means of the CLUSTER algorithm implemented in the SPSS ver 9 package. We followed an iterative method specifying the number of conglomerates from 2 to 30. Different groupings were tested by AMOVA, and that with the least assigned variance within areas was chosen. The data were graphically represented by multidimensional scaling (MDS) plots (Kruskal and Wish 1978) using SPSS.

Qualitative Affinities of Japanese Samples

Particular sharing of subhaplogroups and particular haplotype matches of Japanese samples with concrete Continental areas were phylogeographically analyzed by taking into account the relative genetic diversities of the clades involved in the different areas, measured as relative haplotypic frequencies, and their minimum estimates of coalescence ages based on mean divergence among lineages for the coding region (Saillard et al. 2000). A constant evolutionary rate of 1.7×10^{-8} per site per year (Ingman et al. 2000) was used.

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Parkin attenuates manganese-induced dopaminergic cell death

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Abstract

Manganese as environmental factor is considered to cause parkinsonism and induce endoplasmic reticulum stress-mediated dopaminergic cell death. We examined the effects of manganese on parkin, identified as the gene responsible for familial Parkinson's disease, and the role of parkin in manganese-induced neuronal cell death. Manganese dose-dependently induced cell death of dopaminergic SH-SY5Y and CATH.a cells and cholinergic Neuro-2a cells, and that the former two cell types were more sensitive to manganese toxicity than Neuro-2a cells. Moreover, manganese increased the expression of endoplasmic reticulum stress-associated genes, including parkin, in SH-SY5Y cells and CATH.a cells, but not in Neuro-2a cells. Treatment with manganese resulted in accumulation of parkin protein in SH-SY5Y cells and its redistribution to the perinuclear region, especially aggregated Golgi complex, while in Neuro-2a cells neither expression nor redistribution of parkin was noted. Manganese showed no changes in proteasome activities in either cell. Transient transfection of parkin gene inhibited manganese- or manganese plus dopamine-induced cell death of SH-SY5Y cells, but not of Neuro-2a cells. Our results suggest that the attenuating effects of parkin against manganese- or manganese plus dopamine-induced cell death are dopaminergic cell-specific compensatory reactions associated with its accumulation and redistribution to perinuclear regions but not with proteasome system.

Keywords: dopaminergic cell, endoplasmic reticulum stress, Golgi complex, manganese, parkin, proteasome.

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Metals and pesticides have been the focus of extensive research on the etiology of sporadic Parkinson's disease (PD) as well as environmental parkinsonism (Liou *et al.* 1997; Roman 1998; Smargiassi *et al.* 1998). Manganese (Mn) intoxication causes damage of the substantia nigra (SN) via reduction of tyrosine hydroxylase activity and dopamine (DA) content (Parenti *et al.* 1988; Tomas-Camardiel *et al.* 2002), and is associated clinically with bradykinesia, rigidity and tremor, parkinsonian symptoms (Barbeau 1984; Huang *et al.* 1989; Racette *et al.* 2001). Mn-induced parkinsonism, also known as manganism, is associated with occupational Mn exposures in miners and welders (Rodier 1955; Racette *et al.* 2001). Its pathology is characterized by degeneration of the striatum, especially of the globus pallidum, to lesser extent of the substantia nigra, unlike the degeneration of nigral dopaminergic neurons in idiopathic PD. Recently, Mn toxicity has been reported to activate endoplasmic reticulum (ER) stress-associated genes such as Bip (GRP78) and caspase-12, which are suppressed by overexpression of Bcl-2 and addition of mRNA or protein synthesis inhibitors (Chun *et al.* 2001). These findings suggest that ER stress is in part associated with the Mn-induced parkinsonism.

The *parkin* gene, of which mutations lead to autosomal recessive form of PD, is a member of the E3 ubiquitin ligase (Shimura *et al.* 2000). ER stress caused by accumulation of unfolded protein upregulates parkin mRNA and protein levels, and overexpression of parkin prevents unfolded protein stress-induced dopaminergic cell death (Imai *et al.*

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Abbreviations used: DA, dopamine; ER, endoplasmic reticulum; FITC, fluorescein-5-isothiocyanate; Mn, manganese; PD, Parkinson's disease; PDI, protein disulfide isomerase; SN, substantia nigra; suc-LLVY-MCA, succinyl-LLVY-4-methylcoumaryl-7-amide; TRITC, tetramethylrhodamine; WGA, wheat germ agglutinin; WST-1, 2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium; Z-LLE-β-NA, Z-LLE-β-naphthylamide.

2000). Several recent studies have demonstrated that parkin is also present in Lewy bodies in the SN of non-familial parkinsonism (Choi *et al.* 2000; Schlossmacher *et al.* 2002), suggesting that it is also involved in the pathogenesis of non-familial PD or parkinsonism. However, the role of parkin in Mn-induced parkinsonism is still obscure.

The present study was designed to examine the effects of Mn on parkin and the role of the protein in Mn-induced dopaminergic neuronal cell death. Our results showed that treatment with Mn upregulated parkin protein and resulted in its accumulation in the perinuclear region, together with aggregated Golgi complex, in dopaminergic but not cholinergic cells. Furthermore, overexpression of parkin protected dopaminergic cells from Mn-induced cell death, with or without addition of DA. These results provide a possible mechanism that induction and accumulation of parkin protein are dopaminergic cell-specific compensatory reactions to prevent or ameliorate Mn-induced cell death.

Experimental procedures

Culture of cells

Human dopaminergic neuronal cell line, SH-SY5Y cells (ATCC; #CRL-2266), and mouse cholinergic neuronal cell line, Neuro-2a cells (Japan Health Sciences Foundation, #INFO50081), were cultured at 37°C in 5% CO_2 in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin and 100 μg/mL streptomycin (Gibco-BRL, Rockville, MD, USA). Another dopaminergic CATH.a cells (ATCC; #CRL-11179) derived from mouse DA-containing neurons were cultured at 37°C in 5% CO_2 in RPMI-1640 culture medium (Gibco-BRL) supplemented with 4% fetal bovine serum, 8% horse serum, 100 U/mL penicillin and 100 μg/mL streptomycin. CATH.a cells express DA transporters and susceptible to DA exposure showing elevation of intracellular DA (Higashi *et al.* 2000).

Cell viability analysis

SH-SY5Y cells (6.25×10^4 cells/cm^2), CATH.a cells (5×10^4 cells/cm^2) and Neuro-2a cells (4.5×10^3 cells/cm^2) were plated on each well of a 96-well plate and cultured for 48 h. Then, the cells were treated with 100–800 μM Mn (Sigma Chemical Co., St Louis, MO, USA) for 24 h, followed by simultaneous addition of Mn plus DA hydrochloride (Wako Chemical Co., Hiroshima, Japan) for 24 h (100–200 μM Mn plus 100 μM DA for SH-SY5Y cells; and 100–200 μM Mn plus 50–100 μM DA for Neuro-2a cells). After incubation, the cell viability was assessed by quantitative colorimetric assay with 2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium (WST-1; Dojindo, Kumamoto, Japan), a modification of the standard MTT assay (Higashi *et al.* 2002).

Western blot analysis

SH-SY5Y cells (6.25×10^4 cells/cm^2), CATH.a cells (5×10^4 cells/cm^2) and Neuro-2a cells (4.5×10^3 cells/cm^2) on culture dishes were treated with Mn (100 or 200 μM for CATH.a cells; 200 or 800 μM for SH-SY5Y cells and Neuro-2a cells) for various time intervals. Total cell lysates from Mn-treated cultured were prepared with 10 μg/mL phenylmethylsulfonyl fluoride in ice-cold RIPA buffer [phosphate buffer saline (PBS), pH 7.4, 1% NP-40, 0.5% sodium deoxycholate and 0.1% sodium dodecyl sulfate] or specific lysis buffer to detect Parkin [20 mM HEPES (pH 7.4), containing 150 mM NaCl, 5 mM EDTA, 10% glycerol, 0.5% Triton X-100, 0.5 mM *N*-ethylmaleimide and 0.5 mM iodoacetamide] as described previously (Imai *et al.* 2000). Western blot analyses were performed as described previously (Higashi *et al.* 2002), using goat anti-Bip (Santa Cruz Biotechnology, Santa Cruz, CA, USA; 1 : 200 dilution), mouse anti-protein disulfide isomerase (PDI; Transduction Laboratories, Lexington, KY, USA; 1 : 250 dilution), rabbit anti-caspase-12 (Chemicon, Temecula, CA, USA; 1 : 500 dilution) or rabbit anti-parkin (M74, 1 : 1000 dilution; Shimura *et al.* 1999) polyclonal antibody and corresponding secondary antibody (1 : 5000 dilution) conjugated to horseradish peroxidase. After washing with 20 mM Tris-buffered saline containing 0.1% Tween-20, blots were developed using the ECL western blotting detection system (Amersham Pharmacia Biotech) according to the protocol provided by the manufacturer. Specificity of the detected bands was confirmed by immunoabsorption with each antigen. Incubation of blot using goat anti-actin polyclonal antibody (Santa Cruz Biotech; 1 : 250) normalized sample loading and transfer. The ratio of band intensity (each protein/actin protein) was calculated.

Immunocytochemistry

SH-SY5Y cells (6.25×10^4 cells/cm^2) or Neuro-2a cells (4.5×10^3 cells/cm^2) were plated on four-well chamber slides (Nalge Nunc International Corp., Naperville, IL, USA). After a 48-h attachment period, cells were treated with 800 μM Mn. At 24 h after treatment, the cells were fixed with 4% paraformaldehyde in 0.1 M sodium phosphate buffer. The cells were incubated in 2.5% normal donkey serum and 0.2% Triton X-100 in PBS for 20 min, exposed to rabbit anti-parkin antibody for 18 h, and then reacted with fluorescein-5-isothiocyanate (FITC)-conjugated donkey anti-rabbit IgG antibody (Chemicon) for 2 h. After washing, the cells were incubated with 4 μg/mL tetramethylrhodamine-conjugated wheat germ agglutinin (TRITC-WGA; Sigma) for staining of Golgi complex as described previously (Kubo *et al.* 2001). Observation were made with a confocal laser scanning microscope (excitation 488 nm and emission 505–530 nm for FITC; excitation 543 nm and emission over 560 nm for TRITC).

Analysis of proteasome activity

Proteasome activities after Mn exposure (800 μM) were determined by incubating lysates (5–14 μg of protein) with chymotrypsin fluorogenic substrate succinyl-LLVY-4-methylcoumaryl-7-amide (suc-LLVY-MCA) or post-glutamyl peptidase fluorogenic substrate Z-LLE-β-naphthylamide (Z-LLE-β-NA) for 30 min at 37°C, as previously reported (Keller *et al.* 2000). Background level was determined by incubating lysates with proteasome inhibitor MG115 (20 μM).

Transient transfection assay

After overnight culture of SH-SY5Y cells (6.25×10^4 cells/cm^2) and Neuro-2a cells (4.5×10^3 cells/cm^2) in a 35-mm dish, empty or wild-type human parkin expression vector (1.5 μg for SH-SY5Y cells; 0.8 μg for Neuro-2a cells) was co-transfected with a

pcDNA/Hygro/lacZ plasmid (0.5 μg for SH-SY5Y cells; 0.4 μg for Neuro-2a cells; Invitrogen, San Diego, CA, USA) encoding the β-galactosidase gene into cultured cells using lipofection (Lipofectin; Invitrogen; Higashi *et al.* 2002). At 24 h after the transfection, Mn was added with or without DA (800 μM Mn or 100 μM Mn plus 100 μM DA for SH-SY5Y cells; 800 μM Mn or 200 μM Mn plus 50 μM DA for Neuro-2a cells) for further 24 h, and cells were then stained with 5-bromo-4-chloro-3-indolyl β-galactopyranoside solution as reported previously (Higashi *et al.* 2000).

For transfection with antisense parkin, CATH.a cells (5×10^4 cells/cm^2) cultured overnight on each well of a 96-well plate were transfected with murine parkin antisense cDNA expression vector (0.1 μg/well) or control vector expressing scrambled sequences using lipofection. At 24 h after the transfection, the cells were treated with 50 or 100 μM Mn for further 24 h. After the Mn treatment, cell viability was assessed by trypan blue exclusion assay to count the cell number of trypan blue-exclusion (live) cells.

Statistical analysis

Statistical significance was analysed using one-way or two-way ANOVA, followed by *post-hoc* Fisher's PLSD multiple comparison test.

Results

Manganese-induced cytotoxicity and expression of ER stress-associated molecules

Mn exposure for 24 h induced a dose-dependent cell death of all three cell lines ($F_{8,45} = 29.723$, $p < 0.0001$), and that CATH.a cells and SH-SY5Ycells were more vulnerable to the toxicity of Mn than Neuro-2a cells (Fig. 1a). Because Mn has been reported to induce dopaminergic cell death through activation of ER stress-associated genes (Chun *et al.* 2001), we examined the effects of Mn exposure on expression of ER chaperones, Bip and PDI, and activation of caspase-12 in CATH.a cells. As shown in Fig. 1(b), an increase in Bip expression (1.8-fold) was detected at 24–48 h after 100-μM Mn treatment. The expression of PDI was increased by 5.6-fold within 12-h of Mn treatment, and persisted up to 48 h. Moreover, cleavage of caspase-12 (p40) was observed during the initial 6–24 h.

Effects of manganese on parkin expression

Western blot analysis using anti-parkin antibody showed that parkin protein level was significantly increased by treatment with 100 μM Mn for 48 h (Fig. 1b). At a higher dose of Mn (200 μM), which reduced the number of live cells to 21.3% of control, the induction of these ER stress-associated genes including parkin was also detected in CATH.a cells (data not shown). We also examined the effects of Mn exposure on the ER stress-associated genes in other cell lines, SH-SY5Y cells and Neuro-2a cells. In dopaminergic SH-SY5Y cells, the expression levels of PDI and parkin were also significantly increased 48 h after treatment with Mn at the concentration of 200 μM (data not shown) or 800 μM (Fig. 1c). Whereas,

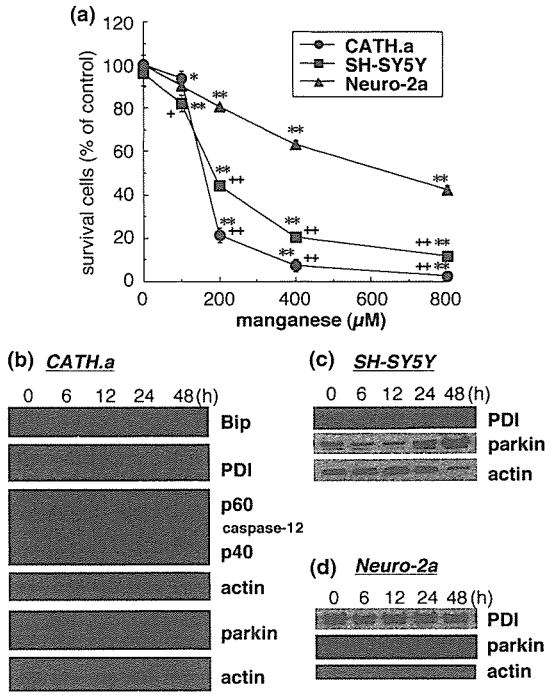

Fig. 1 Effects of Mn on cell viability and expression of ER stress-associated molecules and parkin in dopaminergic and cholinergic cells. (a) CATH.a, SH-SY5Y, and Neuro-2a cells were treated with 100–800 μM Mn for 24 h, and cell viability was measured. Data are the mean ± SEM ($n = 4$). The percentage of surviving cells relative to the number in untreated group is indicated. $^*p < 0.01$, $^{**}p < 0.0001$ compared with each untreated cell group; $+p < 0.05$, $++p < 0.0001$ compared with dose-matched group of Neuro-2a cells. (b) Total cell lysates from CATH.a cells treated with 100 μM Mn for 6–48 h were examined by western blot analysis using anti-Bip, anti-PDI, anticaspase-12 or anti-parkin antibodies. (c and d) Western blot analyses using anti-PDI or anti-parkin antibody in SH-SY5Y cells (c) and Neuro-2a cells (d) treated with 800 μM Mn for 6–48 h.

expression of these indices in non-dopaminergic Neuro-2a cells were not increased by 800 μM Mn (Fig. 1d).

Effects of manganese on intracellular distribution of parkin protein

Parkin protein detected by immunostaining using anti-parkin antibody in naive cells of both SH-SY5Y and Neuro-2a cell lines was observed in the perinuclear region stained with Hoechst 33342 (data not shown), and showed a punctuate distribution along the cell processes and cell bodies similar to the distribution of fluorescent TRITC-WGA signal (Figs 2a and b). Treatment with 800 μM Mn for 24 h, however, markedly increased the parkin immunoreactivity and resulted in accumulation of parkin in the perinuclear region, which was identical to the strong fluorescent signal of TRITC-WGA-positive Golgi complex in SH-SY5Y cells (Figs 2a and c). In contrast, the signal intensity and distribution of

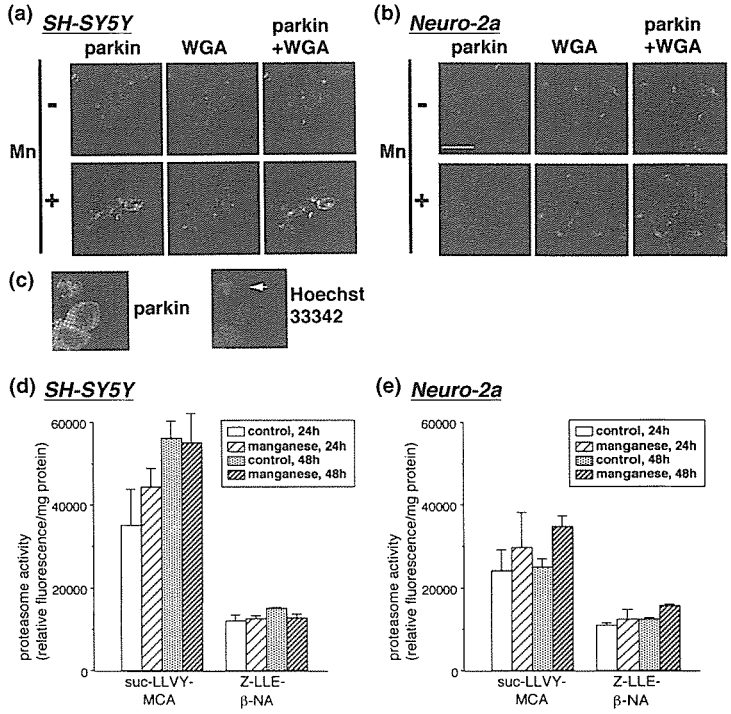

Fig. 2 Effects of Mn on distribution of parkin and proteasome activities in SH-SY5Y and Neuro-2a cells. (a–c) SH-SY5Y cells (a) and Neuro-2a cells (b) treated with 800 μM Mn for 24 h were immunostained using anti-parkin antibody (green), and were stained with 4 μg/mL TRITC-WGA (red). Merged images were obtained by confocal laser scanning microscope program. (c) Representative microphotographs of parkin-immunostaining and corresponding Hoechst nuclear staining of Mn-treated SH-SY5Y cells. (d and e) Chymotrypsin-like and postglutamyl peptidase-like proteasome activities were determined using fluorogenic substrates suc-LLVY-MCA and Z-LLE-β-NA, respectively, at 24 h or 48 h after Mn (800 μM) exposure. Each value is mean ± SEM expressed as the relative fluorescence/mg protein ($n = 4$).

parkin protein were not altered in Neuro-2a cells (Fig. 2b). We also found that a large number of round SH-SY5Y cells, which showed a weak signal for parkin, exhibited chromatin condensation and DNA fragmentation after treatment with 800 μM Mn (data not shown).

Manganese on proteasome activities

We also examined changes in ubiquitin-proteasome system after Mn exposure. Unexpectedly, Mn (800 μM) exposure for 24 h or 48 h did not affect on either proteasome activity examined, chymotrypsin-like activity or post-glutamyl peptidase-like activity, in SH-SY5Y and Neuro-2a cells (Figs 2d and e). Mn (200 μM) showed no changes in either proteasome activities in SH-SY5Y cells (data not shown). Furthermore, co-incubation with proteasome inhibitor lactacystin (10 μM) showed no aggravating effects on Mn-induced cell death in CATH.a cells (data not shown).

Effects of parkin transfection on manganese-induced cytotoxicity

To clarify possible protective effects of parkin against Mn-induced cell death, we performed transient co-transfection of expression vectors encoding parkin and β-galactosidase into SH-SY5Y cells or Neuro-2a cells. In these cells, there was no difference between the number of β-galactosidase-positive cells transfected with parkin expression vector and empty control vector (Figs 3a and b). After exposure to 800 μM Mn for 24 h, the number of β-galactosidase-positive cells transfected with empty vector was reduced to 45.5% and 51.1% of control in both SH-SY5Y cells and Neuro-2a cells, respectively. Parkin-transfected SH-SY5Y cells were significantly resistant to cell death induced by Mn exposure for 24 h (Fig. 3a), but Neuro-2a cells transfected with parkin were not (Fig. 3b). Parkin-transfected CATH.a cells were also significantly resistant to Mn-induced cell death (data not shown). These preventing effects of transfection with parkin expression vector against Mn-induced cell death lasted at later time point, after Mn exposure for 48 h (data not shown).

Treatment with a non-toxic dose of Mn (100 μM) and DA (100 μM) led to marked decrease in the viability of SH-SY5Y cells (31.9% of control) compared with untreated and Mn- or DA alone-treated groups (Fig. 3c) when using the same volume of empty Lipofectin for gene transfection. Even in Neuro-2a cells, non-toxic dose of DA (50 or 100 μM) also significantly enhanced Mn (100 or 200 μM)-induced cell death (Fig. 3d) using lipofection. Furthermore, we examined effects of overexpression of parkin on Mn plus DA-induced neuronal cell death using lipofection. Doses of Mn plus DA were chosen to reduce cell viability to 30–50% of control in each cell line (100 μM Mn + 100 μM DA for SH-SY5Y cells; 200 μM Mn + 50 μM DA for Neuro-2a cells). As shown in Figs 3e and f, overexpression of parkin ameliorated Mn plus DA-induced cell death in dopaminergic SH-SY5Y cells, but not in non-dopaminergic Neuro-2a cells, although Mn plus DA produced cell death in both cell lines.

Effects of antisense parkin transfection on manganese-induced cytotoxicity

Furthermore, Mn (50 or 100 μM)-induced cell death of CATH.a cells was significantly aggravated (− 15.39%,

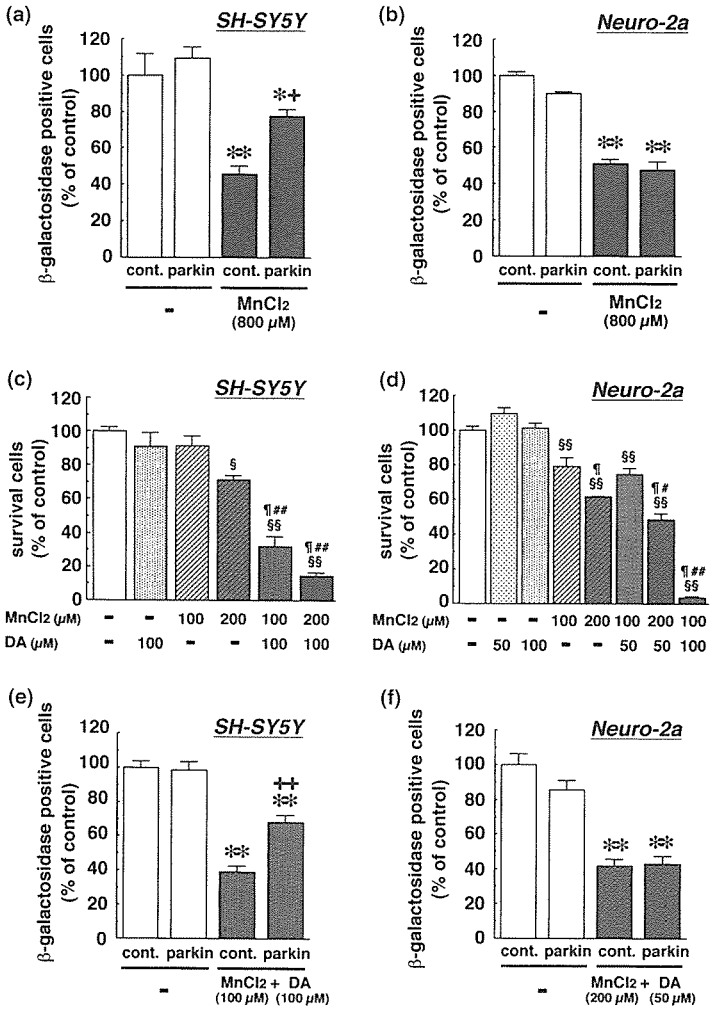

Fig. 3 Effects of overexpression of parkin on Mn- or Mn plus DA-induced cell death of SH-SY5Y and Neuro-2a cells. SH-SY5Y cells (a and e) and Neuro-2a cells (b and f) were transiently transfected with a parkin cDNA expression vector (parkin) or empty vector (cont.) together with a plasmid expressing the β-galactosidase gene using lipofection. At 24 h after transient transfection, cells were treated with 800 μM Mn (a and b) or Mn plus DA (e, 100 μM Mn plus 100 μM DA for SH-SY5Y cells; f, 200 μM Mn plus 50 μM DA for Neuro-2a cells) for further 24 h, and then β-galactosidase-positive cells were stained. Data are mean ± SEM ($n = 3$) and represent the percentages of β-galactosidase-positive cells in untreated empty control vector transfectants. $^{*}p < 0.05$, $^{**}p < 0.001$ compared with untreated empty control vector-transfected, $+p < 0.01$, $++p < 0.001$ compared with treatment-matched empty vector-transfected cells. SH-SY5Y cells (c) and Neuro-2a cells (d) were treated with various doses of Mn plus DA for 24 h using empty Lipofectin, and cell viability was measured. Each value is mean ± SEM ($n = 3$) expressed percentage of surviving cells relative to the number in untreated group. $\S p < 0.01$, $\S\S p < 0.001$ compared with untreated cells; $\P p < 0.001$ compared with the group treated with DA alone; $\#p < 0.05$, $\#\#p < 0.001$ compared with each Mn alone-treated group.

– 16.75% of control, respectively) by transfection with antisense parkin cDNA expression vector (Fig. 4).

Discussion

It has been reported that chronic Mn intoxication in human results in bradykinesia, rigidity and tremor, symptoms typical of PD, and lead to marked degeneration of DA neurons in the SN in monkey (Gupta *et al.* 1980; Huang *et al.* 1989). Furthermore, Mn induces dopaminergic cell death through activation of ER stress-associated genes (Chun *et al.* 2001). In the present study, we examined the effects of Mn exposure on the cell viability and the expression of ER stress-associated genes in dopaminergic SH-SY5Y cells and CATH.a cells as an *in vitro* model of the Mn-induced parkinsonism and also used Neuro-2a cells as a non-dopaminergic control. Mn dose-dependently induced cell death of dopaminergic SH-SY5Y and CATH.a cells and cholinergic Neuro-2a cells. Moreover, Mn increased the expression of ER chaperones, Bip and PDI, and activation of caspase-12 in both dopaminergic cells, but not in non-dopaminergic cells. These results suggest that Mn induced cell death of dopaminergic cells via ER stress. Several neurodegenerative disorders such as Alzheimer's disease are thought to be associated with ER stress (Imaizumi *et al.* 2001; Kouroku *et al.* 2002). Parkin was recently reported to be increased by unfolded protein stress, which contributes to ER stress in SH-SY5Y cells (Imai *et al.* 2000). Therefore, changes in expression of parkin protein were also examined after the Mn treatment. As well as expression of ER chaperones, expression levels of parkin were also significantly increased after the treatment with Mn in dopaminergic CATH.a and SH-SY5Y cells. Whereas expression of these indices in non-dopaminergic Neuro-2a cells were not increased by even high dose of Mn, suggesting that Mn induces ER stress and parkin expression, especially in dopaminergic cells.

The ER stress response leads to increase in ER chaperones such as Bip and PDI to counterbalance the accumulation of unfolded proteins in ER. In human brain, the parkin protein

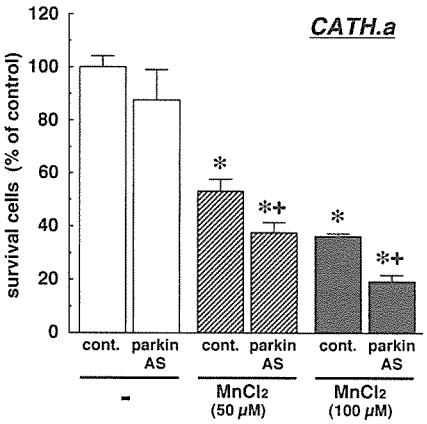

Fig. 4 Effects of transfection with antisense parkin on Mn-induced cell death of CATH.a cells. CATH.a cells were transiently transfected with an antisense parkin cDNA expression vector (parkin AS) or control vector expressing scrambled sequences (cont.) using lipofection. At 24 h after the transfection, cells were treated with 50 or 100 μM Mn for 24 h, and then cell viability was assessed by trypan blue exclusion. Data are mean ± SEM ($n = 4$–5) and represent the percentages of trypan blue-exclusion (live) cells in untreated control vector transfectants. *$p < 0.001$ compared with each untreated cells, +$p < 0.05$ compared with treatment-matched control vector-transfected cells.

is detected in the cytosol and Golgi fractions (Shimura *et al.* 1999). Furthermore, parkin protein is also distributed in the cell bodies and cell processes, and is localized in the perinuclear region together with TRITC-WGA signal of Golgi complex (Kubo *et al.* 2001). Immunostaining using anti-parkin antibody revealed the effects of Mn on subcellular localization of parkin in SH-SY5Y and Neuro-2a cells. In non-treated both cell lines, parkin protein was diffusely distributed in the cytosolic perinuclear region along the cell processes and cell bodies similar to the distribution of Golgi complex, as reported previously (Kubo *et al.* 2001; Junn *et al.* 2002). Treatment with Mn resulted in dramatic accumulation of parkin protein in dopaminergic SH-SY5Y cells and its redistribution to the perinuclear region, especially aggregated Golgi complex, while neither expression nor redistribution of parkin was altered after Mn exposure in non-dopaminergic Neuro-2a cells. Overexpression of parkin has been reported to form aggresome-like perinuclear inclusion in the presence of proteasome inhibitor and to suppress unfolded protein stress-induced cell death (Imai *et al.* 2000; Junn *et al.* 2002). Considered together, we hypothesized that Mn-induced increase in the expression and redistribution of parkin in aggregated Golgi complex seems to protect cells from Mn-induced dopaminergic cell death.

A great number of unfolded proteins in ER is degraded by the ubiquitin-proteasome system, which is known as ER-associated degradation (Hampton 2002). Parkin acts as ubiquitin ligase to ubiquitinate its several substrates (Shimura *et al.* 2000; Imai *et al.* 2001). Unexpectedly, we observed no changes in proteasome activity examined, chymotrypsin-like activity or post-glutamyl peptidase-like activity, in both SH-SY5Y and Neuro-2a cells after Mn exposure in the present study. These resulting data indicate that Mn exposure induces ER stress and aggregation of parkin-positive Golgi complex in dopaminergic cells, but may not affect proteasome function of either dopaminergic or non-dopaminergic cells.

We postulated a possible mechanism that induction and accumulation of parkin protein are dopaminergic cell-specific compensatory reactions to prevent or attenuate Mn-induced cell death. In the present study, transient transfection with parkin expression vector significantly inhibited Mn-induced cell death of dopaminergic SH-SY5Y and CATH.a cells, but not cell death of Mn-treated non-dopaminergic Neuro-2a cells. These results suggest that overexpression of parkin is essential for suppression of Mn-induced dopaminergic cell death. Furthermore, transfection with antisense parkin cDNA aggravated Mn-induced dopaminergic cell death. Extrapolation of these results suggests that parkin may be involved in the protective systems against Mn-induced parkinsonism.

It has been reported that non-toxic concentration of Mn enhances DA- or L-DOPA-induced neuronal cell death (Migheli *et al.* 1999; Stokes *et al.* 2000), and that Mn stimulates DA autoxidation and DA-quinone formation (Donaldson *et al.* 1982; Migheli *et al.* 1999). In both SH-SY5Y and Neuro-2a cells examined in the present study, treatment with a non-toxic dose of DA and low dose of Mn markedly caused and significantly enhanced Mn-induced cell death. We previously reported that toxicity of more higher dose of DA in SH-SY5Y cells is due to extracellular autoxidation of DA, as DA transporter blocker failed to protect cells from DA-induced toxicity (Haque *et al.* 2003). Therefore, extracellular autoxidation of DA through, in part, free radical or quinone generation may enhance Mn toxicity in both SH-SY5Y and Neuro-2a cells examined in the present study. Furthermore, overexpression of parkin attenuated Mn plus DA-induced cell death in dopaminergic SH-SY5Y cells, but not in non-dopaminergic Neuro-2a cells, although Mn plus DA produced cell death in both cell lines. These results suggest that the protective effect of parkin on Mn-induced dopaminergic cell death is not associated with the extrinsic extracellular DA but other intrinsic molecule(s) in the dopaminergic cells. Furthermore, these results in the parkin overexpression strongly support our hypothesis that induction and accumulation of parkin are compensatory reactions, especially in dopaminergic cells, to ameliorate Mn-induced cell death.

Mutations of parkin gene cause neurodegeneration specifically in the SN, although parkin is expressed not only in the SN but also in several brain regions (Shimura *et al.* 1999; Wang *et al.* 2001). In monkeys, Mn intoxication leads to impairment of the nigrostriatal dopaminergic system (Olanow *et al.* 1996). Especially in the dopaminergic cells, but

not in the non-dopaminergic cells, we demonstrated that Mn increased the expression of parkin as well as ER chaperones and resulted in the redistribution of parkin in the aggregated Golgi complex, and that overexpression of parkin prevented Mn-induced cell death. Although parkin can ubiquitinate its potential substrates (Shimura *et al.* 2000; Imai *et al.* 2001), the reported interaction of parkin with those substrates was not specific in dopaminergic neurons. Therefore, the parkin expression on aggregated Golgi apparatus in Mn-treated dopaminergic cells implies a specific involvement of parkin in dopaminergic cell death, and the protective effect of parkin on Mn-induced dopaminergic cells death may involve overexpression and redistribution of parkin. Parkin forms aggresome-like perinuclear inclusion in the proteasome dysfunction (Imai *et al.* 2000; Junn *et al.* 2002). In the present study, Mn induced ER stress and aggregation of parkin-positive Golgi complex in dopaminergic cells, but it showed no alteration in proteasome activities examined. These results suggest that parkin exerts some dopaminergic cell-specific effects which are not related to the ubiquitin-proteasome system in the case of Mn-induced dopaminergic cell death, although further examination will be required to clarify the detail mechanism of ameliorating effects of parkin against Mn-induced toxicity.

Acknowledgements

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日本神経学会賞受賞

パーキンソン病の発症機序：孤発型パーキンソン病研究から家族性パーキンソン病研究へ

服部　信孝

（臨床神経，44：241—262，2004）

序．パーキンソン病の原因を究明に向けて

日本神経学会賞を受賞でき心より感謝するとともにこの賞の重みを感じつつ私どもが推進してきた「パーキンソン病（PD）の発症機序」に関する研究について，今までの順天堂大での研究成果を振り返り，そして21世紀には何をしなければいけないのかを探りながら特別論文として寄稿したい．私がパーキンソン病の研究テーマを水野美邦教授に与えていただいたのは平成元年である．順天堂大は初代教授楢林博太郎先生の時代からわが国でもPD患者が多い屈指の病院として有名であった．正直いって，研究を始める当時，あまりにもPD患者が多く生化学には興味はあったものの神経免疫疾患を研究テーマとして研究に従事していた．水野教授が赴任され神経変性疾患も分子生物学的アプローチが必要であると唱えられ，PD発症の機序に関する研究がスタートした．私は大学院に入学し，水野教授より分子生物学の手ほどきを受けた．回診の合間にミトコンドリアDNAの抽出や免疫組織化学的検討などを大変貴重な時間をとっていただき1つ1つ丁寧に直接指導していただいた．更に水野教授よりミトコンドリア機能異常とPDについての研究を発展させるよう名古屋大生化学第2教室に国内留学させていただいた．この研究生活の経験は大変貴重なものであった．分子生物学の考え方，そしてアプローチの仕方など小澤高将教授，田中雅嗣助教授，鈴木寛講師に鍛えていただいた．再度自分の研究生活を振り返ってみると多くの恵まれた指導者に巡り会うことができた．この名古屋大学時代はむしろデータがまったく出ない冬の時代であったが，名古屋大時代の研究テーマが，後に慶應義塾大の清水信義教授にご教授を受ける機会を与えてくれた．そして常染色体劣性遺伝性パーキンソニズムのパイオニアである山村安弘先生との出会いなど多くの恩師に出会い，そして仕事を発展させることができた．

研究は，十分な成果をもたらしているとはいいがたく，やっとメカニズムの一端が明らかになったに過ぎない．高齢化社会に突入した今世紀は，神経難病克服の時代といわれている．実際，加齢が危険因子であるPDやアルツハイマー病（AD）は今後益々増加する可能性が高く，その予防・治療方法の確立は焦眉の急である．現在PDは10万人当たり100以上の罹患率で，ADに次いで頻度の高い神経変性疾患である．L-dopa療法の発見以来その予後は劇的に改善されつつある一方で，L-dopaの治療経験が積み重ねられるにしたがい長期治療の副作用の問題が明らかにされた．しかしながら，依然として副作用の問題は克服できていない．とくにPDは50〜60歳に発症する患者も多く，その臨床症状は中高年者の生活レベルを低下させるため，大きな社会問題となる要因を孕んでいる．根本治療の開発は不可欠であり，そのために，一次的要因を明らかにすることが最重要課題となっている．われわれは根本治療の開発とくに神経保護作用の薬物治療の開発に向けて10数年来研究をおこなってきた．その研究成果のなかでPDの黒質変性にミトコンドリア機能低下とくに電子伝達系複合体Iの機能低下を報告した．更にこのミトコンドリア研究のなかで常染色体劣性遺伝型を示す家族性PDの原因遺伝子parkinの単離に成功した．単一遺伝子異常にともなう疾患から孤発型疾患へのアプローチはADでもおこなわれており，家族性ADには3種の原因遺伝子が単離され，いずれの遺伝子もアミロイド蛋白の蓄積をひきおこす．遺伝性PDにおいても，病態は共通カスケードを形成している可能性があり，原因遺伝子産物の機能研究が共通メカニズムの解明に繋がる．本論文では，私が水野神経学教室でおこなってきた研究成果について解説したい．

I．孤発型PDにおけるミトコンドリア研究

（1）PDにおけるミトコンドリア機能異常

PDにおけるミトコンドリア機能低下の発見は，1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine（MPTP）によるパーキンソニズムの報告によるところが大きい．このMPTPがミトコンドリア機能低下を惹起させることがわかり，PDにおけるミトコンドリア機能低下が注目されるようになった．その後PDの剖検脳においてミトコンドリア電子伝達系の複合体

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Table 1　Mitochondrial DNA polymorphisms in Parkinson's disease（10 patients）

PD case	Region	Position（n.p.）	Gene	Nucleotide change	Amino acid change
1	Protein-coding gene	4824	ND2	A → G	Thr → Ala
		8794	ATP6	C → T	His → Tyr
	rRNA	663	12SrRNA	A → G	
		1736	16SrRNA	A → G	
		2151	16SrRNA	A → G	
2	Protein-coding gene	14180	ND6	T → C	Tyr → Cyt
	tRNA	15951	tRNA（Thr）	A → G	
3	Protein-coding gene	5301	ND2	A → G	Ile → Val
		12026	ND4	A → G	Ile → Val
	rRNA	752	12SrRNA	C → T	
		1107	12SrRNA	T → C	
		1310	12SrRNA	C → T	
4	Protein-coding gene	4602	ND2	A → G	Thr → Ala
		12358	ND5	A → G	Thr → Ala
	tRNA	4386	tRNA（Gln）	T → C	
5	Protein-coding gene	12358	ND5	A → G	Thr → Ala
		15071	Cytb	T → C	Tyr → His
6	rRNA	1884	16SrRNA	A → G	
	tRNA	4343	tRNA（Gln）	T → C	
		15916	tRNA（Thr）	T → G	
7	Protein-coding gene	4833	ND2	A → G	Thr → Ala
		9165	ATP6	T → C	Phe → Leu
	rRNA	822	12SrRNA	G → T	
	tRNA	5601	tRNA（Ala）	C → T	
		12311	tRNA（Leu）	T → C	
8	rRNA	2772	16SrRNA	C → T	
	tRNA	4386	tRNA（Gln）	A → G	
9	Protein-coding gene	8764	ATP6	G → A	Ala → Thr
		11255	ND4	T → C	Tyr → His
10	Protein-coding gene	13934	ND5	C → T	Thr → Met
	rRNA	827	12SrRNA	A → G	

パーキンソン病10例にみとめられたpolymorphism．Underlineは活性酸素種の攻撃を受けやすいアミノ酸に置換されたものを示す．

I酵素活性の低下が証明された．われわれは剖検脳をもちいて電子伝達系複合体Iのサブユニットが低下していることをWestern blotや免疫組織化学的アプローチで証明した[1)2)]．また酵素活性の検討ではリンパ球をもちいてもミトコンドリア電子伝達系の酵素活性が低下していることがわかった[3)]．更にわれわれはクエン酸回路（TCA回路）の律速酵素と考えられているα-ケトグルタル酸脱水素酵素複合体（α-ketoglutarate dehydrogenase complex；KGDHC）に対する抗体をもちいた免疫組織化学的検討で，PD患者剖検脳においてKDGHCも低下していることを示した[4)]．このミトコンドリア機能低下は，現在では二次的要因として認識されているが，細胞死のカスケードとしてアポトーシスの関与が神経変性疾患でも指摘されており，この細胞内小器官は重要な役割をなしていることはまちがいない．後述するプロテアソーム系は，ATP依存性のプロテアーゼであり，ミトコンドリアのアポトーシスへの関与もふくめて単に呼吸鎖の低下にとどまらず，他の反応系への影響もこの系の低下により避けられないことはまちがいない．

(2) PDのミトコンドリア遺伝子における欠失および点変異

先にふれたようにミトコンドリアは加齢因子の重要な因子であり，ミトコンドリアDNA（mtDNA）の体細胞変異の蓄積が加齢過程や退行性病変において重要な役割をなしていることが推定されており，また多くの確証が存在する．この体細胞変異は，悪性腫瘍や糖尿病においてもその蓄積が報告されている[5)]．

体細胞変異の代表格としては，mitochondrial DNA（mtDNA）の約5kbの欠失がある．この欠失は，PD，対照患者の全例でみとめられるが，半定量をおこなうとPDで欠失mtDNAの蓄積は多く，その蓄積は部位特異性があり前頭葉より線条体で顕著であった[6)]．この体細胞変異の蓄積に，germ line上におけるミトコンドリア遺伝子型がかかわっている可能性がある．そこでPD患者5例のmtDNA全配列を決定したところ，合計55カ所に変異をみいだし，哺乳類の種間で保存されているアミノ酸の置換をひきおこす変異が複数観察された[7)]．更に10例まで拡大して解析したところ，3例のパーキンソン病患者では，ND4遺伝子にTyrをHisに置換する変異とND6遺伝子にTyrをCysに置換する変異がみいだされた（Table 1）[8)]．複合体Iのサブユニットではないが Cytb遺伝子にTyrからHisに置換する変異をみとめた．このHisやCysは活性酸素種の侵襲に感受性が高いといわれ

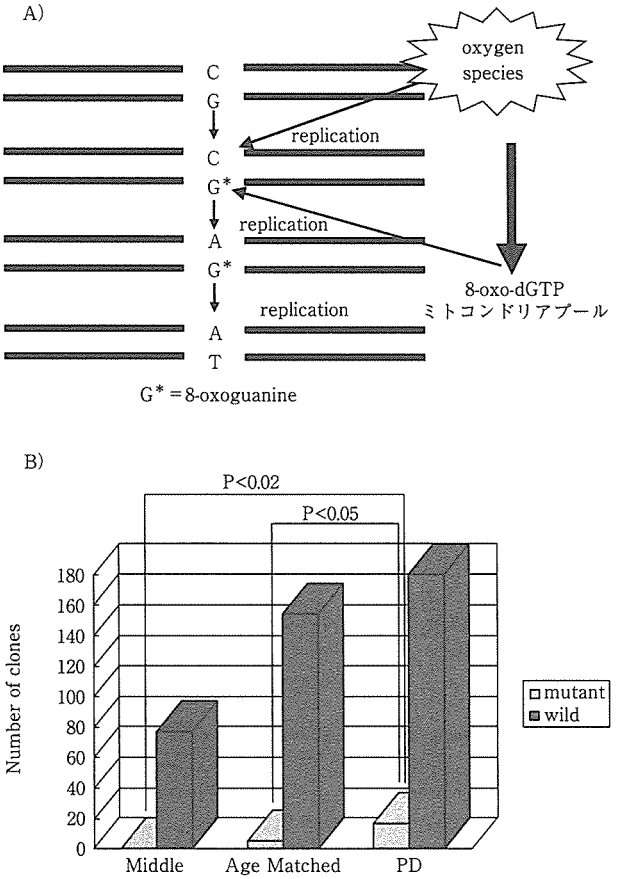

Fig. 1　A：8-oxo-dG は dNTP pool の 8-oxo-dGTP を strand に取り込んでも変異原性となる．8-oxo-dG は C 以外に A ともペアを組むので C：G→A：T transition を生じさせる．B：mtDNA は多重遺伝子なので数コピー存在するような変異 mtDNA を決定することは難しい．Proof reading activity のある *Pfu* polymerase を使い，T vector に cloning して塩基配列を決定した．変異 mtDNA は少なかったが，その頻度は PD で有意に高かった．ターゲットした領域は ND1 で．この部位では複製の際，長い時間一本鎖 DNA の状態で活性酸素種に曝されることが予想される．

ており，これら遺伝子多型による蛋白は，ラジカル脆弱性をもたらすものと予想される．

Shoffner らは tRNAGln における A-to-C transition が PD で有意に頻度が高かったとしているが[9]，われわれは彼らの結果を再現できていない．複数種の遺伝子ついて検討したが有意差を示すような遺伝子多型は存在していなかった．しかしながら，百寿者 64 人と PD について cytochrome b 遺伝子多型である N260D，G251S について両群の頻度を検討してみると百寿者でその頻度が低いことを岐阜県国際バイオ研究所の田中雅嗣先生との共同研究で明らかにした[10]．加齢が重要な因子である PD と健康体である百寿者での，この頻度の違いは mtDNA の variation がより少ないことが成人病に成りにくいことを示していると推定している．

(3) ミトコンドリア遺伝子の体細胞変異の蓄積

mtDNA の複製の特徴として，体細胞変異が蓄積しやすいことは先にふれた．代表格が欠失であるが，点変異型体細胞変異も機能異常を誘発する可能性がある．欠失にしても点変異にしても異常アミノ酸をコードする可能性がある．その結果，異常蛋白が翻訳されることが予想される．ミトコンドリアでは mtDNA と核遺伝子の両遺伝子により一つのユニットである複合蛋白を形成しているため，一個のアミノ酸の違いであっても複合蛋白間内に“揺らぎ”が生じ活性酸素種の発生が増加することが予想される．ではなぜ mtDNA には体細胞変異が蓄積しやすいのか？mtDNA の複製は非対称であり，複製終了には 60 分以上を要する[11]．このことは，一本鎖 DNA の状態が OriL（軽鎖複製点）近傍でもっとも長くなることが予想される．一本鎖 DNA は当然のことながら 2 本鎖 DNA より活性酸素種の攻撃に対して脆弱である．われわれは線条体 mtDNA をもちいて塩基配列の不均一性，つまり体細胞変異として蓄積した塩基置換について分析した．MtDNA を template にした PCR 中の reading error を最小限に押さえる目的で Pfu polymerase をもちい増幅断片をクローン化し，各クローンの塩基配列を決定し，変異頻度を比較した．予想よりは遥かに少なかったが全解析クローン中 8.4% に変異が観察された（Fig. 1，Table 2，3）．一方，中年層では変異を持った mtDNA クローンは存在しなかった．注目すべきことに，これらには終止コドンを生じるナンセンス変異，tRNA の機能的重要な部位の変異がふくまれていた．これらの変異により mtDNA でコードされる遺伝子に病的な機能を獲得した蛋白質が翻訳されることが考えられる．

この体細胞変異によるアミノ酸置換は，複数のサブユニットにより形成される複合体 I では，各サブユニット間の結合に揺らぎが生じ，ミトコンドリア電子伝達系からのラジカル産生が増大することが考えられる．最近になり MNGIE で ND5 領域で欠失生じやすいとの報告があった[12]．この領域は，一本鎖 strand でいる時間帯が必ずしも長くない．われわれが考えている仮説とはことなることになるが，ある特定領域が欠失しやすのは確かなのかもしれない．

(4) PD における核由来ミトコンドリア関連蛋白 24-kDa subunit 遺伝子多型

複合体 I は最低 41 subunits からなる大型の蛋白であり，多くの subunit は核 DNA でコードされる．複合体 I 構成サブユニットの iron sulfur subunit の一つである 24-kDa subunit（NDUFV2）は核由来サブユニットであり，剖検脳の線条体をもちいた Western blot で蛋白量が PD 群で低下していたサブユニットである．まず福井県立大学生物資源の鈴木寛先生との共同研究で NDUFV2 の遺伝子構造をヒト肝由来 cDNA library よりスクリーニングし決定した．NDUFV2 遺伝子は染色体 18 番の短腕に位置する全長約 31.5kb のエクソン 8 個からなる．遺伝子多型のスクリーニングの結果，NDUFV2 の signal peptide をコードしているエクソン 2 に，番号 29 番の Ala から Val に置換している遺伝子多型をみいだした[13]．アレル頻度は対照群と有意差はなかったが，genotype で分析すると，ホモ接合体は PD 群で有意に高かった．この変異は，C 末端付近で α ヘリックス構造が β シート構造に変化することでミトコンドリア内でのシグナルペプチドのプロセッシング効率が低下することが推定されている．日本人で

Table 2 Summary of human mtDNA analyzed

Subject	Age	Sex	Diagnosis	A	B	C
PD patients						
P1	65	F	PD	62	4	49,600
P2	77	M	PD	60	5	48,000
P3	72	M	PD	58	5	46,400
Total						144,000
Controls						
C1	38	M	Malignant lymphoma	27	0	21,600
C2	47	F	Gastric cancer	24	0	19,200
C3	55	M	Renal failure	25	0	20,000
Subtotal				76		60,800
C4	64	M	Cervical spondylosis	37	0	29,600
C5	70	F	Laryngeal carcinoma	56	2	44,800
C6	78	M	Heart failure	61	2	48,800
Subtotal				154		123,200
Total						184,000

A, number of clones analysed ; B, number of mutant clones observed ; C, number of nucleotides analysed totaly from each of individuals.
PCR 産物を T-vector に cloninng して塩基配列を決定した．Wild との比較で変異のクローンの数を解析した．

Table 3 Amino acids changes due to nucleotide substitutions observed in present study and its conservation among six mammalian species

Subject	Position	Amino acid change	Rat	Mouse	Bovine	Seal	Whale	Human	Patient
PD Patients									
P1	3543	Leu to Leu	L	L	L	L	L	L	L
	3316	Ala to Thr	I	I	I	I	I	A	T
P2	3316	Ala to Thr	I	I	I	I	I	A	T
	3543	Leu to Leu	L	L	L	L	L	L	L
	3457	Asp to Asn	D	D	D	D	D	D	D
P3	3497	Ala to Val	L	L	A	L	A	A	V
	3338	Val to Ala	I	V	I	I	L	V	A
	3506	Thr to Asn	S	S	S	S	S	T	N
	3467	Lys to Stop	K	K	K	K	K	K	Stop
Controls	3316	Ala to Thr	I	I	I	I	I	A	T
	3380	Arg to Gln	R	R	R	R	R	R	Q
	3443	Leu to Pro	L	L	L	L	L	L	P
	3543	Leu to Leu	L	L	L	L	L	L	L
All clones	3423	Val to Val	N	I	V	I	I	V	V

観察された変異の一部は mammalian で保存されているアミノ酸部位に存在し，stop codon に置換されているものがあった．

有意差をみとめたが，白人では有意差がなく人種により結果がことなる．更に症例数を増やして検討する必要がある．

われわれは，更にドパミン代謝に関与する Catechol-O-methyltransferase 遺伝子多型についても検討をおこなったが[14]，人種により頻度がことなり，MtDNA，核 DNA ふくめて AD の ApoE 遺伝子の4型のような発症を促進させるようなリスクファクターは今のところはっきりしない．遺伝子関連分析では，人種などによりその頻度がことなり，評価が難しい．後述する UCH-L1 遺伝子多型だけは，人種差を超えて保護的作用が存在する可能性がある．

(5) パーキンソン病におけるミトコンドリア遺伝子防御酵素

活性酸素は mtDNA 中のデオキシグアノシン（dG）を 8-オキソデオキシグアノシン（8-oxo-dG）へ変化させる potential を持っており，8-oxo-dG は複製の際にアデニンに誤読される．この 8-oxo-dG が，パーキンソン病患者脳で対照脳に比して，組織特異的に黒質で有意に増加しているとの報告がある．われわれは脂質過酸化の代謝産物である 4-hydorxy-2-nonenal（HNE）が，PD で有意に上昇していることを免疫組織化学的に証明しており[15]，PD の病態に酸化ストレスが関与していることはまちがいないと考えている．更にこの HNE 修飾蛋白は Lewy 小体内にも存在していることが報告され