leukocyte count $\geqslant 100 \times 10^9$/l, or 10 years old or older with leukocyte count $\geqslant 50 \times 10^9$/l (assigned to auto-SCT group), autologous blood or marrow SCT or chemotherapy could be elected. Each institute declared the choice in advance of the study initiation.

Statistical analysis

The duration of EFS was defined as the time from the initiation of therapy to the date of failure (that is any relapse, death, or diagnosis of secondary malignancy) or to the date when patients were confirmed to be in remission and alive. Patients who did not achieve complete remission at the end of the initial induction phase or who died before the confirmation of remission were considered to have failed at day 0, even if they entered remission later with a second course or through additional treatment. The probability of EFS and s.e. was estimated by the Kaplan–Meier method (Greenwood), and differences were tested by the log-rank test. Analysis was performed with the intent to treat. 'Any central nervous system (CNS) relapse' include both 'isolated CNS relapse' and CNS relapse combined with other sites. Probability of cumulative CNS relapse was estimated by inversed Kaplan–Meier method, which involves subtraction of Kaplan–Meier products from 100%. Only patients who had CNS relapse were failure, and all the others were censored. Cumulative probability of any secondary malignancy was calculated using the same method. Patients who received modified treatment were censored at that point in time. The patients who did not enter complete remission or had died during induction were treated as at the date of the beginning of treatment. Patients who were confirmed as remaining in first remission and alive, or who were lost of follow-up, were censored for EFS analysis; all those who were alive with or without disease were censored in OS analysis at the date of last contact.

Follow-up was updated in 2008. The proportions of patients whose data of the last 5 years were available were 144 of 357 (40.3%) in L84-11 study, 197 of 306 (64.3%) in L89-12, 220 of 266 (82.7%) in L92-13, and 449 of 489 (91.8%) in L95-14.

Results

Probability of EFS, OS, and cumulative CNS relapse rate of each study are shown in Tables 1 and 3. There was no improvement in EFS during the first three studies. The OS of L92-13 improved,

Table 3 Summary of the study results

Studies	*L84-11*	*L89-12*	*L92-13*	*L95-14*
Number of eligible patients (B+T)	484	418	347	597
Number of B/T	420/32	375/43	315/32	539/58
Average age (B/T) year	5.7/8.8	5.9/8.2	5.8/7.7	5.9/7.7
Average WBC (B/T)	20.1/108.0	31.6/137.5	38.4/146.1	30.6/167.0
Number of censored early	0	1 (0.2%)	2 (0.6%)	9 (1.5%)[a]
Death during induction	3 (0.6%)	12 (2.9%)[b]	5 (1.4%)	10 (1.7%)[c]
Failure of initial remission	11 (2.3%)[d]	17 (4.1%)[e]	5 (1.4%)	11 (1.8%)[f]
Complete remission (rate)	470 (97.1%)	388 (92.8%)	335 (96.0%)	567 (95.0%)
Corrected remission (rate)[g]	477 (98.6%)	399 (95.7%)	337 (97.7%)	573 (97.4%)
Death in first remission	19 (3.9%)	7 (1.7%)	6 (1.7%)	22 (3.7%)[h]
Number of censored in first remission	13 (2.7%)	13 (3.1%)[i]	31 (8.9%)[j]	21 (3.5%)[k]
Number of patients at event free	308 (63.6%)	256 (61.2%)	180 (55.3%)	428 (71.7%)
Number of relapse after remission	123 (26.1%)	104 (26.9%)	112 (33.4%)	92 (16.7%)
Site of relapse: total	123 (100%)	104 (100%)	112 (100%)	92 (100%)
Isolated bone marrow (BM)	72 (58.5%)	70 (67.3%)	87 (78.4%)	68 (73.9%)
Isolated CNS	17 (13.8%)	13 (12.5%)	3 (2.7%)	10 (10.9%)
Isolated testis	19 (15.4%)	6 (5.8%)	9 (78.4%)	7 (7.6%)
BM+CNS	6 (4.9%)	4 (3.8%)	3 (7.2%)	5 (5.4%)
BM+testis	7 (5.7%)	7 (6.7%)	6 (3.6%	1 (1.1%)
CNS+testis	1 (0.8%)	1 (0.9%)	0	0 (0%)
Other sites	1 (0.8%)	3 (2.9%)	3 (2.7%)	1 (1.1%)
Secondary AML/MDS	0/1	3/1	0/0	2/1
Brain tumor/Other	5/1[l]	4	2	1
Any BM	85 (69.1%)	81 (77.9%)	97 (87.4%)	74 (80.4%)
Any CNS	24 (19.5%)	18 (17.3%)	6 (5.4%)	15 (16.3%)
Any testis	27 (22.0%)	14 (13.5%)	15 (13.3%)	8 (8.7%)
Any testis/males	27 (10.3%)	14 (5.8%)	15 (8.5%)	8 (2.4%)

Abbreviations: AML, acute myeloid leukemia; CNS, central nervous system; MDS, myelodysplastic syndrome; SCT, stem-cell transplantation; WBC, white blood cells.
[a]Four patients assigned in dexamethasone arm dropped off, one in prednisolone arm, and four in HR risk group dropped off.
[b]Marrow suppression and infection.
[c]Five deaths in dexamethasone arm, two deaths in prednisolone arm, three deaths in HR risk.
[d]7/11 entered into remission in the following phase.
[e]11/17 patients entered remission in the following phase.
[f]All 11 failures in HR risk group; 3 Ph+ALL, 4 chromosomal translocations, 6/11 entered into remission in the following phase.
[g]Corrected remission (rate %): patients who achieved delayed remission were included in remission, and censored patients during the induction phase were excluded from the total.
[h]18/22 deaths in HR risk group, 5 related with transplants.
[i]7/13 patients underwent SCT in CR1.
[j]26/31 patients underwent SCT in CR1.
[k]9/21 patients underwent SCT in CR1.
[l]Olfactory neuroblastoma.

compared with these of the earlier two studies. The L95-14 study achieved internationally acceptable level of EFS and OS (log-rank $P<0.0001$). The cumulative 'any CNS relapse' rate decreased from 5.5% (any CNS) in the L84-11 study to 2.8% in the L95-14 study.

Twelve treatment-related brain tumors developed in patients who had received cranial irradiation in the four studies—that is 5, 4, 2, and 1 patient, respectively. They developed in six males and six females. No brain tumor occurred in the non-irradiated patients. The tumors developed between 8 and 22 years after cranial irradiation, seven in the 18-Gy irradiated group and five in the 24-Gy irradiated group. The probability of cumulative incidence (±s.e.) of brain tumors was 1.9±0.6% at 15 years and 2.8±0.9% at 20 years among the 1234 irradiated patients. Secondary acute myeloid leukemia (AML)/myelodysplastic syndrome (MDS) developed in eight patients—that is 0/1, 3/1, 0, and 2/1 in each study. Two of them (L89-12) were confirmed to have 11q23 chromosome abnormality. Seven of the eight patients were female, whereas brain tumors developed evenly in terms of gender. AML/MDS occurred only in the irradiated patients without exception. The probability of cumulative incidence±s.e. of AML/MDS among irradiated patients was 0.57±0.25% at 3 years and 1.1±0.4% at 10 years.

Cerebrovasucular lesions such as Moyamoya disease developed after radiation in the TCCSG studies and published elsewhere.[18] Neurocognitive evaluation study was not carried out as a group.

Protocol-specific treatment result

L84-11 study. For 484 patients enrolled, EFS±s.e. and OS±s.e. were 66.3±2.2 and 73.5±2.1% at 15 years, respectively. There were 357 long-term survivors, and their median follow-up period was 16.6 years. Among survivors, seven had serious neurological sequelae, such as paraparesis or leukoencephalopathy, which developed most probably because of cranial irradiation and concentrated use of five TITs at body-surface-adjusted dose setting. Probability of cumulative incidence of brain tumors in L84-11 was 1.2±0.7% at 15 years (Tables 3 and 4; Figure 1).

Males fared significantly worse than females in terms of EFS (Table 4; $P=0.006$), but not in terms of OS ($P=0.205$). Isolated or combined testicular relapses developed in 27 out of 261 males (10.3%) and they comprised 22% of all relapses.

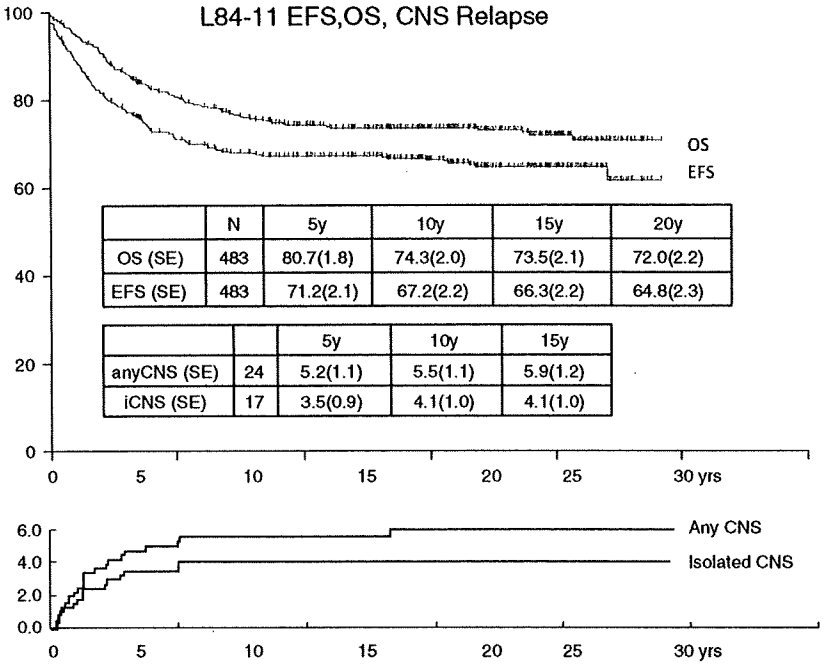
As a result of the randomized comparison in SR, the EFS±s.e. rates of the S1 and S2 arms were 68.5±4.8 and 81.0±4.1%, respectively, at 15 years (log-rank test, $P=0.071$). The probabilities of cumulative incidence ±s.e. of any testicular relapse were 24.3±6.7% in S1 arm and 4.7±3.3% in S2 arm (log-rank $P=0.015$).

L89-12 study. For the 418 patients enrolled, the EFS±s.e. and OS rate were 62.3±2.6 and 71.9±2.2% at 1 year, respectively. Probability of cumulative isolated CNS and any

Table 4 Treatment results according to presenting features in non-infant patients treated in study L84-11

Factors	*Number of patients*	*Event-free survival ± s.e.%*				*Overall survival ± s.e.%*			
		5 years	*10 years*	*15 years*	*log-rank P-value*	*5 years*	*10 years*	*15 years*	*log-rank P-value*
Non-T lineage									
NCI standard	314	72.8±2.5	69.4±2.6	68.5±2.7	0.074	83.4±2.1	77.6±2.4	77.2±2.4	0.012
NCI high	106	67.6±4.7	61.0±4.9	59.0±5.1		73.6±4.4	66.1±4.8	64.8±5.0	
T-lineage									
NCI standard	9	55.6±16.6	44.4±16.6	44.4±16.6	0.636	66.7±15.7	55.6±16.6	41.7±17.3	0.487
NCI high	23	60.9±10.1	60.9±10.1	60.9±10.1		65.2±9.9	65.2±9.9	65.2±9.9	
Sex									
Male	261	66.4±3.0	61.3±3.2	60.8±3.1	0.006	80.1±2.5	72.1±2.8	71.1±2.9	0.205
Female	222	78.1±3.0	74.5±3.0	73.1±3.1		81.5±2.6	76.9±2.9	76.4±2.9	
Age at diagnosis (years)									
1–9	392	72.6±2.3	69.2±2.4	68.5±2.4	0.068	82.7±1.9	76.5±2.2	75.9±2.2	0.007
⩾10	91	65.0±5.2	58.7±5.3	56.8±5.5		72.0±4.8	64.7±5.1	63.2±5.2	
WBC × 10^9/l									
<10k	265	76.5±2.6	73.1±2.8	71.9±2.9	0.0131	86.4±2.1	80.9±2.5	80.4±2.5	0.002
10–49k	159	64.6±3.9	59.7±4.0	5.9±4.0		75.8±3.4	67.5±3.8	66.0±3.9	
50–99k	31	63.5±8.8	56.0±9.2	56.0±9.2		70.0±8.3	58.4±9.3	58.4±9.3	
⩾100k	28	67.9±8.8	67.9±8.8	67.9±8.8		67.3±9.0	67.3±9.0	67.3±9.0	
Cell lineage									
Non-T	420	71.5±2.2	67.3±2.3	66.3±2.4	0.121	81.0±1.9	74.7±2.2	74.1±2.2	0.038
T	32	59.4±8.7	55.9±8.8	55.9±8.8		65.6±8.4	62.2±8.6	58.5±8.1	
TCCSG risk arms									
S1	102	74.4±4.4	69.9±4.7	68.5±4.8	0.071	91.0±2.9	83.1±3.8	79.6±5.1	0.227
S2	93	85.7±3.7	81.0±4.1	79.1±4.5		94.5±2.5	87.3±3.6	87.3±3.6	
H1	129	69.8±4.1	67.2±4.2	66.0±4.3	0.131	77.7±3.7	73.4±4.0	71.4±4.1	0.046
H2	113	62.7±4.6	57.5±4.8	57.5±4.8		70.9±4.3	61.9±4.7	61.9±4.7	
S1 testis	49	21.8±6.4	24.3±6.7	24.3±6.7	0.009				
S2 testis	50	2.3±2.3	4.7±3.3	4.7±3.3					

Abbreviations: NCI, National Cancer Institute risk group; s.e., standard error; TCCSG, Tokyo Children's Cancer Study Group, WBC, white blood cells.
Testis: probability of cumulative any testicular relapse rate in males.



	N	5y	10y	15y	20y
OS (SE)	483	80.7(1.8)	74.3(2.0)	73.5(2.1)	72.0(2.2)
EFS (SE)	483	71.2(2.1)	67.2(2.2)	66.3(2.2)	64.8(2.3)

		5y	10y	15y
anyCNS (SE)	24	5.2(1.1)	5.5(1.1)	5.9(1.2)
iCNS (SE)	17	3.5(0.9)	4.1(1.0)	4.1(1.0)

Figure 1 EFS, OS, and cumulative incidence of isolated or any CNS relapses in L84-11 study.

CNS relapse rates were 3.7 ± 1.1 and 5.4 ± 1.3% at 15 years, respectively. Of the 306 surviving patients, the median survival period was 14.6 years. Secondary neoplasms consisted of four brain tumors, three AML, and one MDS. Remission induction rate was 92.8%, which was the lowest of the four studies (Table 3). Twelve patients (2.9%) died during or after the remission induction course, between days 10 and 82. The major cause of death was prolonged marrow suppression and infection. Of 17 patients (4.1%) failed to enter remission at the end of induction, six patients (1.4%) died within 4–24 months; one Ph positive ALL, and four with leukocyte count $>145 \times 10^9$/l. The other 11 patients entered remission in the following phase; five patients with leukocyte counts $>100 \times 10^9$/l, seven Ph positive ALL. The corrected remission rate was 95.7% when the patients who entered into delayed remission were included in remission and those who were dropped off during induction were excluded from the total number. Pirarubicin used for induction at a dosage of 30 mg/m^2 (two or three doses) was amended to 20 mg/m^2 in October 1990. Nine out of 12 deaths occurred before the amendment. Testicular relapse was significantly fewer in incidence in SR0 (HD-MTX) arm than the SR18 arm ($P=0.018$; Tables 3, 5; Figure 2).

L92-13 study. EFS ± s.e. and OS ± s.e. for 347 eligible patients enrolled were 60.1 ± 2.7 and 77.9 ± 2.2% at 10 years, respectively. Cumulative rate of isolated CNS relapse was 1.0 ± 1.0 at 10 years, which might be underestimated by high bone marrow relapse rate. The median follow-up period was 13.0 years for the 271 (78.1%) patients remaining alive, including 64 patients who experienced relapse. Twenty-one HR patients underwent hematopoietic SCT at first remission (treated as censored), and 18 were alive in CR (Tables 3, 6; Figure 3).

Brain tumors occurred in two patients. No myeloid leukemia or MDS developed. The rate of remission induction was 96.0%.

Seven of 26 relapses among 62 males in SR group relapsed very late at 5–13 years of the initial therapy, whereas females stopped recurring at 5 years. Overall, the EFS in males was 47.5 ± 4.3% at 15 years, which was significantly lower than that in females (68.0 ± 3.8%, $P=0.0003$). Males were, however, more efficiently salvaged. The OS of males was 75.8 ± 3.3% and that of females 80.3 ± 3.1% ($P=0.731$; Table 6). Ten of 14 patients with isolated or combined testicular survived. After relapse, 51 patients survived out of 84 who had undergone hematopoietic SCT (actual survival 60.7%). Of 25 who had been treated with chemotherapy, 15 survived after relapse (60%). The OS rate of 77.4 ± 2.4% eventually exceeded the preceding two studies.

L95-14 study. L95-14 study achieved 5-year EFS ± s.e 75.0 ± 1.8% and the OS ± s.e. 82.0 ± 1.6%, at 10 years' follow-up. For the 489 patients who remained alive, the median follow-up period was 10.0 years. The remission induction rate after the initial course was 95.0%. The corrected remission induction rate was 97.5% when nine patients who were off during induction were excluded and six patients who entered into remission in the following phase were included. The cumulative isolated CNS relapse rate was 1.7 ± 0.6% and 'any CNS relapse' rates was 2.8 ± 0.7% for all patients, and the latter level was 4.3 ± 1.4% in the HR. One brain tumor occurred at 8.3 years, two AML, and one MDS all were diagnosed between 1.5 and 5.2 years of therapy (Tables 3, 7; Figures 4).

The results of randomized control study was updated and showed again no advantage of DEX arm over PSL arm in SR and IR groups[7] (Tables 2, 7). Three extramedullary relapses occurred in the DEX arm, whereas eight developed in the PSL arm.

Hematopoietic SCTs, either allogeneic or autologous blood and marrow source, were elected by institutional intention to

Table 5 Treatment results according to presenting features in non-infant patients treated in study L89-12

Factors	*Number of patients*	*Event-free survival ± s.e.%*				*Overall survival ± s.e.%*			
		5 years	*10 years*	*15 years*	*log-rank P-value*	*5 years*	*10 years*	*15 years*	*log-rank P-value*
Non-T lineage									
NCI standard	314	72.8±2.5	69.4±2.6	68.5±2.7	0.074	83.4±2.1	77.6±2.4	77.2±2.4	0.012
NCI high	106	67.6±4.7	61.0±4.9	59.0±5.1		73.6±4.4	66.1±4.8	64.8±5.0	
T-lineage									
NCI standard	11	70.1±14.7	70.1±14.7	70.1±14.7	0.169	70.1±14.7	70.1±14.7	70.1±14.7	0.369
NCI high	32	51.9±9.0	51.9±9.0	43.3±10.9		55.3±8.9	55.3±8.9	55.3±8.9	
Sex									
Male	240	62.1±3.2	59.8±3.3	57.8±3.4	0.044	76.3±2.8	72.2±3.5	71.1±3.0	0.564
Female	178	74.1±3.4	70.8±3.5	68.3±3.7		79.6±3.1	75.2±3.3	73.0±3.5	
Age at diagnosis (years)									
1–9	320	70.8±2.6	68.0±2.7	66.6±2.7	0.0002	81.8±2.2	78.3±2.4	77.5±2.4	<0.0001
⩾10	97	54.3±5.3	51.6±5.4	46.2±5.7		64.2±4.9	57.5±5.1	53.0±5.4	
WBC × 10^9/l									
<10k	203	75.5±3.1	70.7±3.4	67.8±3.5	<0.0001	88.1±2.3	83.5±2.7	81.5±3.0	<0.0001
10–49k	133	67.7±4.1	66.0±4.2	66.0±4.2		77.5±3.7	73.5±3.9	72.7±3.9	
50–99k	31	47.1±9.1	43.5±9.1	43.5±9.1		61.2±8.7	54.8±8.9	51.4±9.0	
⩾100k	50	44.4±7.2	44.4±7.2	40.0±7.7		46.7±7.2	44.6±7.2	44.6±7.2	
Cell lineage									
Non-T	374	68.3±2.5	65.2±2.6	63.3±2.6	0.053	79.8±2.1	75.0±2.3	73.3±2.4	0.009
T	43	57.1±7.7	50.7±9.1	50.7±9.1		59.1±7.7	59.1±7.7	59.1±7.7	
CNS status									
CNS blast +	12	42.9±15.7	42.9±15.7	42.9±15.7	0.132	56.3±14.8	46.9±15.0	46.9±15.0	0.033
CNS blast–	406	68.1±2.4	65.0±2.4	62.8±2.5		78.3±2.1	74.2±2.2	72.6±2.3	
TCCSG SR arms									
SR0	83	75.4±4.9	72.7±5.1	72.7±5.1	0.399	90.6±3.4	89.2±3.6	87.7±3.9	0.148
SR18	64	71.5±5.7	66.5±6.0	66.5±6.0		85.8±4.4	80.9±5.0	78.1±5.5	
SR0 CNS	83	5.4±2.6	—	—	0.999				
SR18 CNS	64	5.2±2.9	—	—					
SR0 testis	83	3.3±3.3	—	—	0.018				
SR18 testis	64	19.4±7.1	22.9±7.6	—					

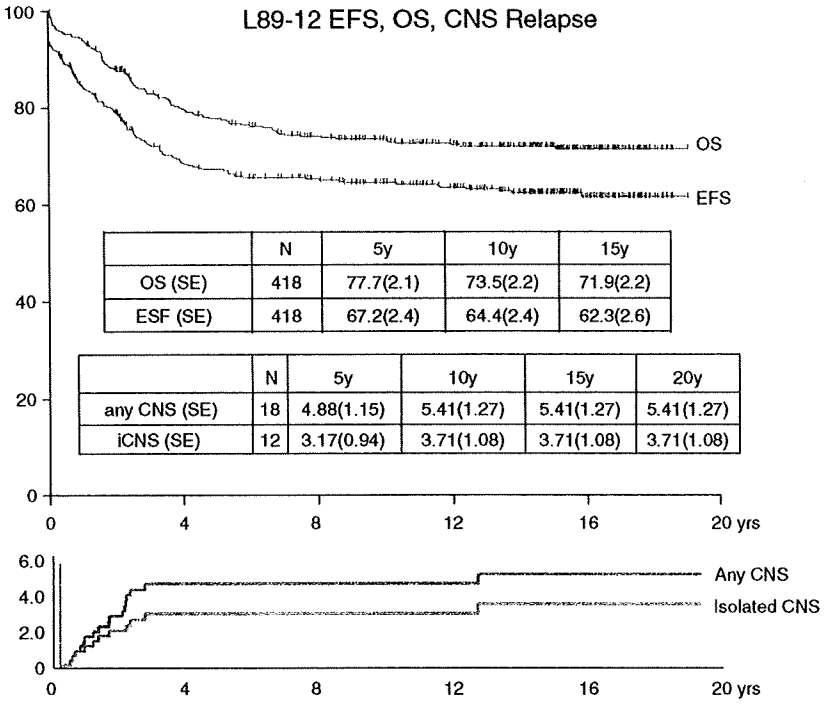
Abbreviations: CNS, central nervous system; NCI, National Cancer Institute risk group; s.e., standard error; SR, standard risk; TCCSG, Tokyo Children's Cancer Study Group; WBC, white blood cells.
CNS: probability of cumulative any CNS relapse rate.
Testis: probability of cumulative any testicular relapse rate.

treat decision in advance and executed for 61 (37 allo-SCT and 24 auto-SCT) of 126 patients who assigned to SCT (59 allo-SCT and 67 auto-SCT), among which 44 (actual rate72.1%) were alive without relapse. Of the 65 patients who assigned to SCT group, but elected chemotherapy, 30 (46, 2%) patients were alive; 29 were in first remission.

Treatment results according to presenting features

Well-documented prognostic factors were analyzed in each of the four studies (Tables 4–7). Infants were not included in these studies. Patients with B-precursor ALL and T-ALL were analyzed separately in each of the four studies, according to the NCI / Rome criteria. Age and leukocyte count at diagnosis were still independently strong prognostic factors.

Patients with T-ALL had poor prognosis. This was more evident in terms of OS (Tables 2–5). Clearly, patients with T-ALL could not be easily salvaged after relapse. Females fared significantly better than males in terms of EFS at 10 years by 13.2 points (L84-11, $P=0.006$), 11.0 points (L89-12, $P=0.044$), 15.6 points (L92-13, $P=0.003$), and –2.8 points (L95-14, males fared better, $P=0.519$), respectively (Table 3). 'Any testicular relapse' rate was 10.3, 5.8, 8.5, and 2.4% of all the males in the four studies, respectively (Table 3). The cumulative incidence of testicular relapse was significantly lower in ID-MTX or HD-MTX arms in randomized trials of the L84-11 SR, L89-12 IR, and L92-13 IR, as has been described.[19] The gender difference in EFS correlated well with the incidence of testicular relapse. Approximately 60% of the patients with any testicular relapse survived and contributed to the recovery of male OS to the same level as females. CNS involvement at presentation had negative prognostic impact on EFS (Tables 4 and 5). In L95-14 study (Table 7), patients who presented with DNA index of 1.16–1.60 showed EFS 84.2±3.5%, which was significantly higher than the EFS rate of 72.3±2.2% among those with DNA index<1.16 ($P=0.005$).[20] DNA index 1.16–1.60 group of patients also fared better than those with DNA index over 1.6 (EFS of 50.0±17.7%, $P=0.003$).The outcome of the patients with Ph chromosome was dismal. Hematopoietic SCT was only curative treatment strategy so far.[21]



	N	5y	10y	15y
OS (SE)	418	77.7(2.1)	73.5(2.2)	71.9(2.2)
ESF (SE)	418	67.2(2.4)	64.4(2.4)	62.3(2.6)

	N	5y	10y	15y	20y
any CNS (SE)	18	4.88(1.15)	5.41(1.27)	5.41(1.27)	5.41(1.27)
iCNS (SE)	12	3.17(0.94)	3.71(1.08)	3.71(1.08)	3.71(1.08)

Figure 2 EFS, OS, and cumulative incidence of isolated or any CNS relapses in L89-12 study.

Discussion

Nine years passed since the earlier issue was published in '*Leukemia* 2000.'[1] The 1423 survivors in the four studies are now 22.5 years old on an average, ranging from 11.6 to 39.8 years of age. Of 1233 patients who received cranial irradiation, 873 were surviving. Twelve secondary brain tumors developed very late, that is at 8–22 years after initial therapy including cranial irradiation in the four studies presented here. The development of the brain tumors seemed not to depend on the studies. Hijiya *et al.*[22] reported from the St Jude that the cumulative incidence of brain tumor except for meningioma was 3.00 ± 0.59% at 30 years. It was 2.8 ± 0.9% at 20 years in the four studies.

As for the secondary AML/MDS, the incidence was variable depending on the study. They developed only in the irradiated patients without exception. Regimens of L89-12 and L92-13 studies included etoposide, which is a topo-II inhibitor and was highly associated with the development of secondary MAL/MDS with 11q23 chromosome translocations.[23,24] Two cases were confirmed to be associated with chromosome 11q23 translocations in L89-12 study. It was noteworthy that seven out of eight secondary AML/MDS patients were female, whereas the brain tumors developed equally across genders. It was described that girls were more sensitive to anthracycline cardiac toxicity than boys.[25] In addition, cognitive impairment, short stature, and excessive weight were all more prevalent among females than males.[26] Females responded more to the chemotherapy and remained in higher EFS than that of males. All these facts may suggest that girls are more sensitive to anti-leukemic drugs, resulting in better outcome of ALL and developed more therapy-related secondary AML/MDS.

Schmiegelow recently reported from NOPHO studies that children with low thiopurine methyltransferase activity were at lower risk of relapse of ALL[27] and were at higher risk of developing secondary malignancy.[28] In the latter article, of 20 secondary malignancies, 16 AML/MDS occurred in 6 males and 10 females, although the author did not mention the gender difference.

We had not performed neurocognitive assessment as a group, but many studies showed the negative influence of the cranial irradiation on the neurocognitive function particularly for the young patients,[26] and other study described that normal neurological function was preserved when irradiation was omitted.[29]

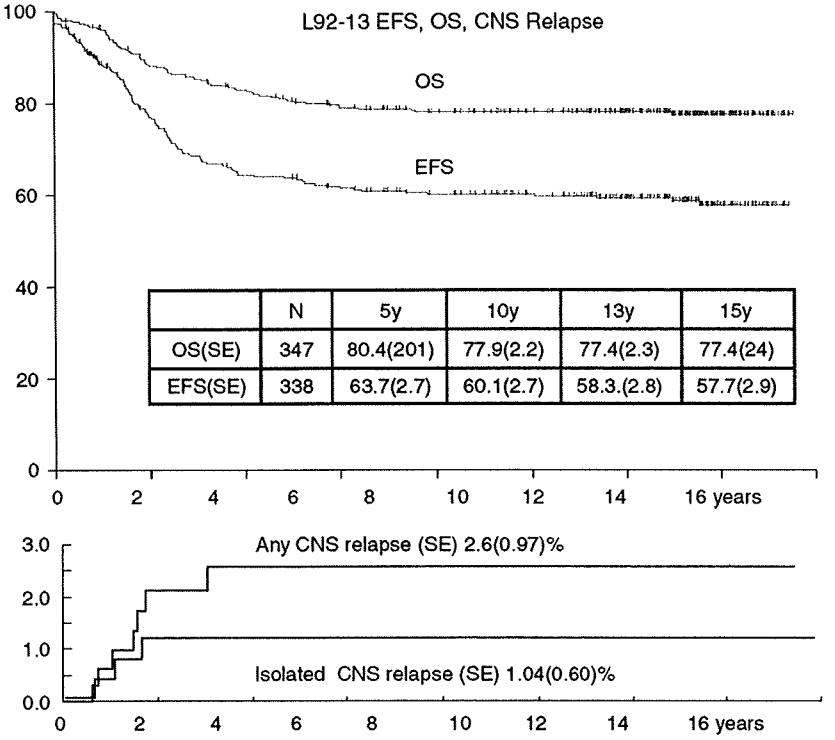
In the next study of TCCSG ALL L99-15, irradiated patients were limited to <10%. In the currently active study, T-ALL and prednisolone poor responders were irradiated. The outcomes have already been reported on the protocols with no cranial irradiation from St Jude Children's Research Hospital,[30] EORTC,[31] Nordic countries,[32] and Netherlands.[33] To eliminate the cranial irradiation, the function of intrathecal injections would be expected. The 9–11 times intrathecal injections ended before 40 weeks in TCCSG protocols even when no cranial irradiation was administered. The proper number and timing of the extended intrathecal injections for patients at risk of CNS relapse such as hyper-leukocytosis and T-ALL remained to be determined in our future studies.

Gajjar *et al.*[34] express strong caution to traumatic lumber punctures as a risk factor of CNS relapse. The L89-12 and L92-13 studies had 1-week prophase of single therapy with oral prednisolone, and the initial intrathecal injection and cerebrospinal fluid examination was given on day 8.[5,35] The prednisolone prophase without spinal puncture might well have alleviated cerebrospinal fluid infiltration before the assessment. Consequently, initial ratio of patients with CNS-2 or CNS-3 was

Table 6 Treatment results according to presenting features in non-infant patients treated in study L92-13

Factors	*Number of patients*	*Event-free survival ± s.e.%*				*Overall survival ± s.e.%*			
		5 years	*10 years*	*15 years*	*log-rank P-value*	*5 years*	*10 years*	*15 years*	*log-rank P-value*
Non-T lineage									
NCI standard	206	68.1 ± 3.3	64.0 ± 3.4	62.8 ± 3.4	0.01	88.7 ± 2.2	86.1 ± 2.4	86.1 ± 2.4	<0.0001
NCI high	108	56.5 ± 5.1	52.9 ± 5.1	52.9 ± 5.1		68.1 ± 4.5	64.9 ± 4.7	64.9 ± 4.7	
T-lineage									
NCI standard	7	83.3 ± 15.2	83.3 ± 15.2	83.3 ± 15.2	0.132	100	100	100	0.062
NCI high	25	50.8 ± 11.4	50.8 ± 11.4	50.8 ± 11.4		60.0 ± 9.8	60.0 ± 9.8	60.0 ± 9.8	
Sex									
Male	177	56.2 ± 3.9	52.4 ± 3.9	47.5 ± 4.9	0.003	80.5 ± 3.0	77.0 ± 3.0	75.8 ± 3.3	0.731
Female	170	71.3 ± 3.6	68.0 ± 3.7	68.0 ± 3.8		80.3 ± 3.0	80.3 ± 3.1	80.3 ± 3.2	
Age at diagnosis (years)									
1–9	264	66.4 ± 3.0	62.7 ± 3.1	59.7 ± 3.3	0.025	86.7 ± 2.1	84.7 ± 2.3	84.0 ± 2.4	<0.0001
⩾10	83	55.0 ± 5.8	51.7 ± 5.9	51.7 ± 5.10		67.7 ± 5.2	55.2 ± 5.3	55.2 ± 5.4	
WBC × 10^9/l									
<10k	164	65.9 ± 3.4	60.6 ± 3.9	59.9 ± 11.1	0.302	85.2 ± 2.8	82.7 ± 3.0	82.7 ± 3.1	0.008
10–49k	109	79.1 ± 4.0	64.5 ± 4.7	58.1 ± 5.4		81.5 ± 3.7	78.6 ± 4.0	77.1 ± 4.2	
50–99k	21	65.3 ± 10.6	59.9 ± 11.0	59.9 ± 11.1		76.2 ± 9.3	78.6 ± 4.0	77.1 ± 4.2	
⩾100k	50	53.9 ± 7.8	53.9 ± 7.8	53.9 ± 7.9		63.7 ± 6.8	63.7 ± 6.8	63.7 ± 6.8	
Cell lineage									
Non-T	315	64.1 ± 2.8%	60.3 ± 2.9%	57.6 ± 3.1%	0.779	81.6 ± 2.2%	78.9 ± 2.3%	78.2 ± 2.4%	0.177
T	32	58.5 ± 9.8%	58.5 ± 9.9%	58.5 ± 9.10%		68.7 ± 8.2%	68.7 ± 8.3%	68.7 ± 8.4%	
CNS status									
CNS-1	323	65.5 ± 2.8	61.7 ± 2.9	60.8 ± 2.9	0.525	80.9 ± 2.2	79.2 ± 2.4	78.5 ± 2.1	0.128
CNS-2	12	55.0 ± 15.0	55.0 ± 15.0	55.0 ± 15.0	0.076*	66.7 ± 13.6	58.3 ± 14.2	58.3 ± 14.2	
CNS-3	9	37.5 ± 17.1	37.5 ± 17.1	37.5 ± 17.1		88.9 ± 10.5	88.9 ± 10.5	88.9 ± 10.5	
DNA index or chromosome number (50–60 or others, others include cases not tested)									
1.16–1.60	25	68.0 ± 9.3	52.0 ± 10.0	52.0 ± 10.0	0.775	92.0 ± 5.4)	92.0 ± 5.4)	92.0 ± 5.4)	
Others	322	63.0 ± 2.8	60.5 ± 2.8	59.0 ± 2.9		78.5 ± 2.3)	76.9 ± 2.4)	76.3 ± 2.4)	
t(9;22) or BCR/ABL chimera message									
Present	12	16.7 ± 10.8	-	-	<0.0001	33.3 ± 13.6	33.3 ± 13.6	33.3 ± 13.6	<0.0001
Absent	335	64.6 ± 3.0	61.0 ± 2.8)	60.2 ± 2.8		82.1 ± 2.1	79.6 ± 2.2	79.0 ± 2.3	
TCCSG arms									
SR	123	65.9 ± 4.3	59.9 ± 4.5	56.3 ± 4.6		88.3 ± 2.9	84.9 ± 3.3	83.5 ± 3.5	
IR0	71	61.0 ± 5.9	58.0 ± 6.0	58.0 ± 6.0	0.942	87.1 ± 4.0	87.1 ± 4.0	87.1 ± 4.0	0.021
IR18	50	64.0 ± 6.8	60.0 ± 6.9	60.0 ± 6.9		74.0 ± 6.2	69.9 ± 6.5	69.9 ± 6.5	
IR0 testis	37	7.8 ± 5.5	7.8 ± 5.6	7.8 ± 5.7	0.053				
IR18 testis	22	26.4 ± 10.2	26.4 ± 10.3	26.4 ± 10.4					

Abbreviations: CNS, central nervous system; CSF, cerebrospinal fluid; NCI, National Cancer Institute risk group; s.e., standard error; SR, standard risk; TCCSG, Tokyo Children's Cancer Study Group; WBC, white blood cells.
IR0: the arm without cranial irradiation.
IR18: the arm with cranial irradiation.
Testis: probability of cumulative any testicular rate in males.
*CSF-1 vs CSF2 + 3.



	N	5y	10y	13y	15y
OS(SE)	347	80.4(201)	77.9(2.2)	77.4(2.3)	77.4(24)
EFS(SE)	338	63.7(2.7)	60.1(2.7)	58.3.(2.8)	57.7(2.9)

Figure 3 EFS, OS, and cumulative incidence of isolated or any CNS relapses in L92-13 study.

lower on day 8 in our studies than that on day 1 of other studies. It has been shown that the day 8 puncture did not increase CNS relapse.[5] The initial day 8 lumbar puncture is a safe method to avoid inadvertent introduction of leukemic blasts into the cerebrospinal fluid.

The duration of the maintenance therapy had been shortened step by step from 4 years in L81-10 study, 3 years for SR in L84-11 study, and 1.5 years for SR and 1 year for HR in L89-12 study without increasing relapses. The ID-MTX in S2 arm of L84-11 study efficiently reduced relapse after off therapy, whereas the control arm showed clusters of relapse starting at the point of off therapy. These results developed a hypothesis that an addition of a new intensified treatment on early phase might make it possible to shorten the duration of therapy further without sacrificing overall outcome. Randomized study could not be realized because a control arm was difficult to set. For the intensification of early therapy, ID-CA and HD-CA and mitoxantrone were administered in all risk groups. As a result, the relapse increased in both SR and HR groups. The short maintenance therapy affected more negatively on the lower-risk patients and males than on the higher risk and females (Table 6). EFS of HR patients was almost equivalent to that of SR. The early intensification might be more effective in HR than SR as CCG reported.[36] Randomized comparison of length in maintenance therapy for 18 months vs 24 months came to conclusion in ALL-BFM 81[4] and 83[37] studies, and ALL-BFM 86[38] study was amended to extend all the maintenance from 18 to 24 months. The appropriate length of maintenance therapy must be essential, particularly for the lower-risk patients and males. The duration between 18 months and 24 months were needed in the protocols of BFM-type structure. The boys had a higher risk of late relapse without sufficient maintenance therapy.

In 95-14, the randomized study in SR and IR compared between prednisolone (60 mg/m^2 at induction and 40 mg/m^2 at intensifications) and dexamethasone (8 mg/m^2 at induction and 6 mg/m^2 at intensifications) resulted in no significant difference in EFS rate.[7] Analysis with updated data on this comparison resulted in the same conclusion. Our results did not fully accord with those of other larger-scale studies. The results of CCG-1922 study[39] showed significantly better outcome in SR patients treated with dexamethasone at 6 mg/m^2 than prednisolone 40 mg/m^2. In UK Medical Research Council ALL97 trial,[40] dexamethasone given at 6.5 mg/m^2 and prednisolone given at 40 mg/m^2 were compared, and the dexamethasone arm showed better outcome. A conclusive result is anticipated in the trials with higher dose of dexamethasone at 10 mg/m^2 along with the evaluation of side effects.

In conclusion, analysis of long-term follow-up results brought us invaluable suggestions to consider for our future studies. Girls may generally be more drug sensitive than boys and they could be cured with shorter maintenance therapy than boys; at the same time, they may be at higher risk of secondary AML/MDS. The testicular relapse and lower EFS in boys were almost resolved in L95-14. TCCSG currently limited the indication of cranial irradiation to <10% of the patients. To avoid the secondary malignancy and neurological sequelae, it is of primary importance to omit the cranial irradiation and the etoposide completely as a primary therapy. Safe and effective induction and immediately given intensification, as well as appropriate length of maintenance therapy, are still major subjects to study. We seriously realized that an establishment of firm long-term follow-up system is mandatory to evaluate the ultimate result of the protocols.

Table 7 Treatment results according to presenting features in non-infant patients treated in study L95-14

Factors	Number of patients	Event-free survival ± s.e.%				Overall survival ± s.e.%			
		5 years	10 years	13 years	log-rank value	5 years	10 years	13 years	log-rank P value
Non-T lineage									
NCI standard	373	82.7 ± 2.0	81.3 ± 2.1	80.5. ± 2.2	<0.0001	92.5 ± 1.3	90.6 ± 1.5	88.9 ± 2.0	<0.0001
NCI high	183	67.4 ± 3.6	64.4 ± 3.7	64.4 ± 3.7		72.8 ± 3.3	68.5 ± 3.6	67.3 ± 3.7	
T-lineage									
NCI standard	8	87.5(11.7)	87.5(11.7)	87.5(11.7)	0.2676	100	100	100	0.095
NCI high	50	66.9 ± 6.8	66.9 ± 6.8	66.9 ± 6.8		70.0 ± 6.5	68.0 ± 6.6	68.0 ± 6.6	
Sex									
Male	340	78.5 ± 2.6	76.5 ± 2.7	76.5 ± 2.7	0.519	86.1 ± 2.1	84.4 ± 2.3	82.9 ± 2.7	0.211
Female	257	75.4 ± 2.4	73.7 ± 2.5	72.9 ± 2.6		84.0 ± 2.0	80.1 ± 2.2	78.7 ± 2.4	
Age at diagnosis (years)									
1–9	460	79.1 ± 1.9	77.6 ± 2.0	77.0 ± 2.1	0.002	88.6 ± 1.5	85.7 ± 1.7	83.8 ± 2.0	<0.0001
⩾10	134	68.6 ± 4.1	65.6 ± 4.3	65.6 ± 4.3		72.4 ± 3.9	69.2 ± 4.1	69.2 ± 4.1	
WBC × 10^9/l									
<10k	306	79.1 ± 2.3	77.2 ± 2.4	75.7 ± 2.6	<0.0001	91.1 ± 1.6	88.0 ± 1.9	86.52 ± 2.4	<0.0001
10–49k	160	74.8 ± 3.4	74.1 ± 3.4	74.1 ± 3.4		86.7 ± 2.7	85.3 ± 2.8	85.3 ± 2.8	
50–99k	58	56.9 ± 6.5	56.9 ± 6.5	56.9 ± 6.5		70.7 ± 6.0	65.8 ± 6.6	62.3 ± 7.1	
⩾100k	70	57.7 ± 6.0	55.6 ± 6.1	55.6 ± 6.1		65.4 ± 5.7	65.4 ± 5.7	65.4 ± 5.7	
Cell lineage									
Non-T	539	77.5 ± 1.8	75.5 ± 1.9	75.3 ± 2.0	0.159	86.1 ± 1.5	83.4 ± 1.7	81.4 ± 1.9	0.021
T	58	69.7 ± 6.2	69.7 ± 6.2	69.7 ± 6.2		73.9 ± 5.8	72.1 ± 5.9	72.1 ± 5.9	
CNS status									
0	378	85.6 ± 1.8	82.3 ± 2.0	81.8 ± 2.1	0.962	77.9 ± 2.0	77.9 ± 2.0	77.9 ± 2.0	0.514
1–4	183	85.1 ± 2.6	83.9 ± 2.7	80.2 ± 3		77.5 ± 3.0	74.7 ± 3.0	74.7 ± 3.0	
5–	20	90.0 ± 6.7	77.9 ± 9.9	77.9 ± 9.9		65.8 ± 11.0	65.8 ± 11.0	65.8 ± 11.0	
DNA index									
<1.16	464	74.3 ± 2.1	72.9 ± 2.1	72.3 ± 2.2	0.005*	82.5 ± 1.8	79.2 ± 2.9	78.2 ± 2.0	0.001*
1.16–1.60	124	87.5 ± 3.0	84.2 ± 3.5	84.2 ± 3.5	0.003**	94.3 ± 2.1	92.7 ± 2.4	92.7 ± 2.4	0.005**
>1.60	9	50.0 ± 17.7	50.0 ± 17.7	50.0 ± 17.7		77.8 ± 13.9	77.8 ± 13.9	77.8 ± 13.9	
t(9;22) or BCR/ABL chimera message									
Present	24	26.4 ± 9.7	26.4 ± 9.7	26.4 ± 9.7	<0.0001	41.7 ± 10.1	31.3 ± 9.9	25,9 ± 9.7	<0.0001
Absent	573	78.7 ± 1.7	76.9 ± 1.8	76.4 ± 1.9		86.8 ± 1.4	84.1 ± 15.7	83.9 ± 1.8	
t(1;19) or E2A/PBX1 chimera message									
Present	26	70.2 ± 9.5	70.2 ± 9.5	70.2 ± 9.5	0.449	73.0 ± 8.7	73.0 ± 8.7	73.0 ± 8.7	0.182
Absent	568	77.1 ± 1.8	75.1 ± 1.9	74,7 ± 1.9		85.5 ± 1.5	82.4 ± 1.6	80.8 ± 1.9	
11q23 or MLL rearrangement									
Present	5	75.0 ± 21.5	75.0 ± 21.5	75.0 ± 21.5	0.962	80.0 ± 17.9	80.0 ± 17.9	80.0 ± 17.9	0.879
Absent	589	76.8 ± 1.8	74.9 ± 1.8	74.5 ± 1.9		85.0 ± 1.5	82.0 ± 1.6	80.5 ± 1.8	
TCCSG SR+HR arm									
Dexamethasone	179	82.15 ± 2.9	80.5 ± 3.1	80.5 ± 3.1	0.5178	91.5 ± 2.1	89.1 ± 2.4	88.1 ± 2.6	0.190
Prednisolone	180	85.6 ± 2.7	83.5 ± 2.9	81.9 ± 3.2		95.0 ± 1.6	93.2 ± 1.9	90.2 ± 3.5	

Abbreviations: CNS, central nervous system; CSF, cerebrospinal fluid; NCI, National Cancer Institute risk group; s.e., standard error; SR, standard risk; TCCSG, Tokyo Children's Cancer Study Group; WBC, white blood cells.
*<1.16 vs 1.16-1.60, **1.16-1.60 vs>1.60.

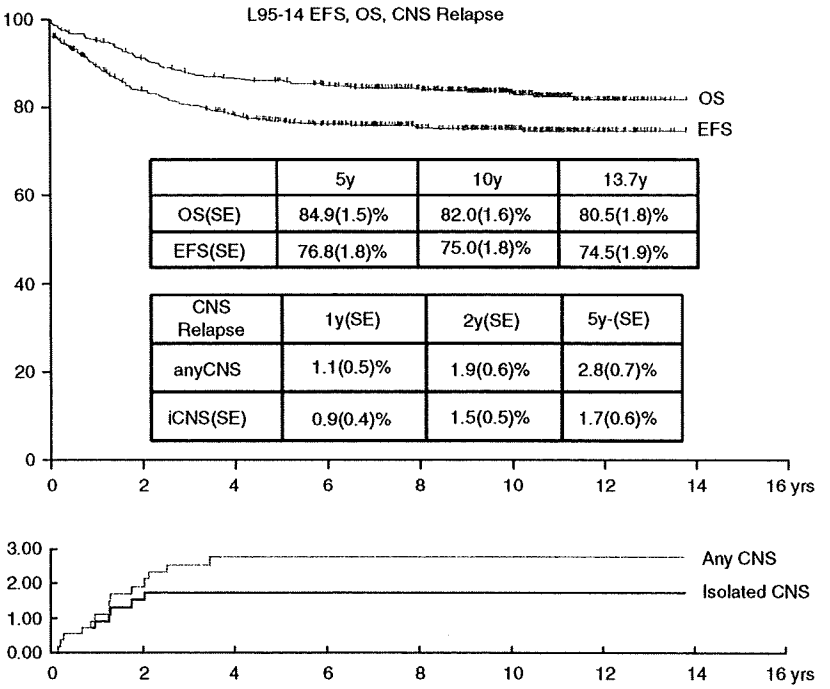


Figure 4 EFS, survival, and cumulative incidence of isolated or any CNS relapses in L95-14 study.

Conflict of interest

The authors declare no conflict of interest.

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Expression of Bone Morphogenetic Proteins in Giant Cell Tumor of Bone

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Abstract. *Background: A giant cell tumor (GCT) of bone is a locally aggressive tumor with a propensity for local recurrence. A characteristic pattern of peripheral bone formation has been described in GCT recurrence in soft tissue, and in some pulmonary metastases from benign GCT. Although the bone formation in GCT in supposedly due to bone morphogenetic proteins (BMPs), the expression pattern of BMPs in GCT has not been well investigated. Materials and Methods: The expression of BMPs in GCT tissues, cultured stromal cells from GCT, and osteoclast-like giant cells harvested by laser microdissection (LM), as well as from control osteosarcoma (NOS-1) cells was analyzed using reverse transcriptional-semiquantitative PCR. Results: BMP 2, 3, 4, 5 and 6 were expressed in the GCT tissue. The cultured GCT cells expressed BMP 2, 4, 5 and 6. The osteoclast-like giant cells expressed BMP 2, 3, 5 and 6 and BMP 5 was expressed at the highest level. Conclusion: Both stromal cells and osteoclast-like cells in GCT expressed several kinds of BMPs.*

A giant cell tumor (GCT) of bone is a distinctive locally aggressive neoplasm of undifferentiated cells. The multinucleated osteoclast-like cells apparently result from the fusion of mononuclear cells. Apart from such multinucleated giant cells, there are also two mononuclear cell types in GCT. The first has a round morphology similar to monocytes. The second cell type is spindle-shaped, fibroblast-like stromal cells (1-3). Cell culture experiments with GCT cells have revealed stromal cells to be the proliferating component of the GCT. The stromal cells probably stimulate monocyte migration in to the tumor tissue and enhance their fusion into the osteoclast-like giant cells (4-9). The giant cell itself resembles a normal osteoclast that is able to resorb bone, thus leading to extended osteolysis. Bone morphogenetic proteins (BMPs) are morphogens capable of inducing new cartilage and bone in ectopic sites. The bone forming cells (osteoblasts and osteocytes) produce BMPs (10). The proteins act as autocrine and/or paracrine factors regulating bone growth and remodeling. Recently BMP expression has also been demonstrated in osteoclasts by immunohistochemical analyses. In addition, the bone morphogenetic activity was observed in GCT tissue (11-22). Recurrence in soft tissue of GCT is a rare complication, and the tendency for these lesions to ossify is unexpected, given the lack of significant bone formation in primary or recurrent intraosseous lesions. A characteristic pattern of peripheral bone formation has been described in GCT involving soft tissue recurrence, and in some pulmonary metastases from benign GCT (1-3). This suggests that, in the extraosseous environment, the cells from GCT are able to simulate osteoblastic differentiation and bone formation. In addition, a bioassay for bone formation activity of lyophilized bone tumors has indicated that human GCT has bone morphogenetic activity, and both immunohistochemical and Western blotting studies have revealed the expression of BMP in GCT (23, 28-30). However, the expression pattern of BMPs in GCT has not yet been fully characterized.

This article describes the relationship between the expression of BMPs and the cell types in GCT.

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Key Words: Giant cell tumor, osteoclast-like giant cell, stromal cell, bone morphogenetic protein, laser microdissection.

Table I. *PCR primers.*

Transcript	Sense primer	Antisense primer
TRAP	5'-AAGGAGGACTACGTGCTCGTGGCCCGGC-3'	3'-TCCACTCAGCACGTAGCCCACGCCGTT-5'
BMP2	5'-TCCTCTCATCAGCCATTTGTCCTTC-3'	3'-AGTTACTACACATTCTTCATAG-5'
BMP3	5'-TCAAATGAGTTCTTTGCCAGGTTATC-3'	3'-CGCCAGGAGATACCTCAAGGTAGA-5'
BMP4	5'-ACCTGAGACGGGGAAGAA A-3'	3-TTA AAGAGGAAACGAAAAGCA-5'
BMP5	5'-AAGAGGACAAGAAGGACTAAAAATAT-3'	3'-GTAGAGATCCAGCATAAAGAGAGGT-5'
BMP6	5'-CTGGGTAATAAGGCACTGGCATG-3'	3'-GTCGTAATCGCTCTACCCAGTCC-5'
BMP7	5'-AGATAGCCATTTCCTCACCG-3'	3-TGGAGCACCTGATAAACGCT-5'

TRAP: Tartrate-resistant acid phosphatase.

Materials and Methods

RT-PCR of fresh tumor tissue and cultured cells. Fresh giant cell tumors tissue specimens were obtained from surgical patients who all provided their informed consent. Each tissue specimen was chopped into small pieces and then was placed on dishes containing Roswell Park Memorial Institute tissue culture medium (RPMI) 1640 supplemented with 10% heat-inactive fetal bovine serum, and 300 mg kanamycin sulfate (Wako Tokyo, Japan). These were maintained in a humidified atmosphere of 5% CO_2 in air at 37°C. The fresh frozen giant cell tumor tissue and the spindle-shaped adherent cultured cells after three to five passages (1×10^6 cells) were harvested for RNA extraction using guanidine isothiocyanate/cesium chloride gradient centrifugation. A human osteosarcoma cell line (NOS 1) which had prominent osteoinductive activity (23) was used as a positive control. The RNA was then reverse transcribed to cDNA using 100 units of Moloney murine leukemia virus reverse transcriptase per reaction with an oligo-dT primer (Promega, Madison, WI USA). The oligonucleotide primers have been described in previous reports (Table I) (23-25). The BMP 2, BMP 5 and BMP 7 primers were designed in house and the specific amplification was confirmed by a direct sequencing analysis using the dideoxy-chain termination method employing an ABI Prism 310 genetic analyzer and Big Dye Terminator cycle sequencing ready reaction kit (Perkin Elmer, Foster City, CA, USA).

Semi-quantitative PCR. The reaction mixture had a total volume of 20 μl containing 2.0 μl 10×PCR buffer (TOYOBO, Osaka Japan), 25 mM $MgCl_2$, 2 mM deoxynucleotide-triphosphates, 1 μl of each BMP primer and 0.2 μl of β-actin or GAPDH primers. The primer pairs for specific genes and β-actin or glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) were included in the same tube for coamplification. After PCR, the amplified sequences were separated on a 2% agarose gel and visualized by staining with ethidium bromide. The intensity of the bands was measured by NIH Image Ver 1.63 (developed at US National Institutes of Health and available by anonymous FTP at http://rsb.info.nih.gov/nih-image/download.html), and the levels of mRNA of BMP 2, BMP 3, BMP 4, BMP 5, BMP 6 and BMP7 relative to the level of GAPDH and β-actin mRNAs were calculated (26).

RT-PCR of giant cells. Frozen sections (5 μm) of fresh giant cell tumor tissues were made and covered mounted on glass slides covered with crosslinked polyethylene (PEN) foil (2.5 μm thick; Leica Microsystem, Wetzlar, Germany). The sections were fixed with methanol at –20°C for 10 s, and thoroughly air dried. Thereafter, the sections were washed with diethylpyrocarbonate (DEPC)-treated water, stained with 0.05% toluidine blue (TB) solution, (Wako Pure Chemical Industries, Ltd., Osaka, Japan) for 10 s, and then the TB solution was rinsed out with DEPC-treated water, and thereafter the sections were dried. The giant cells were dissected from the frozen sections with a laser microdissection (LMD) system using a 337-nm nitrogen ultraviolet (UV) laser (*Leica* Laser Microdissection System, Leica Microsystems) Figure 1. The dissected giant cells were dropped immediately into a microcentrifuge tube cap filled with 10 μl XB buffer (Picopure RNA Isolation Kit, Arcturus, CA, USA). Over 1,000 giant cells from an individual patient were collected into a 0.5 ml tube, and then the total RNA was extracted using a Picopure RNA Isolation Kit. RT-PCR was performed as described above. Each sample was quantified in triplicate in each of three separate PCR reactions.

Results

The individual tissue samples are described in Table II. The RT-PCR results were expressed as the percentage of mRNA in comparison to two housekeeping genes (β-actin, GAPDH).

The percentages of BMP 2, 3, 4, 5, 6 and 7 mRNA relative to the housekeeping gene transcripts in the control (NOS-1) cells were: BMP 2, 99.6±13.8%; BMP 3, 34.9±4.9%; BMP 4, 47.3±8.9%; BMP 5, 1.8±0.3%; BMP 6, 52.5±2.2% and BMP 7, 56.9±9.9%; Figure 2-A). BMP 2, 3, 4, 5 and 6 were expressed in the GCT tissue (Figure 2-B). A relatively high level of expression of BMP 2, 5 and 6 was detected, and the BMP 5 expression is illustrated in Figure 3A. The cultured cells expressed BMP 2, 4, 5 and 6. A relatively high level expression of BMP 6 was detected. The mean BMP 6 expression in the cultured cells was 33.6% in comparison to the housekeeping gene expression (Figure 2-C). The osteoclast-like multinucleated giant cells expressed BMP 2, 3, 5 and 6. There was a relatively high level of expression of BMP 5 (Figure 3-A) and 6 detected. The mean BMP 5 expression was 62.9% in comparison to the housekeeping genes (Figure 2-D). No BMP 7 expression was detected in the GCT tissue, cultured cells or osteoclast-like giant cells.

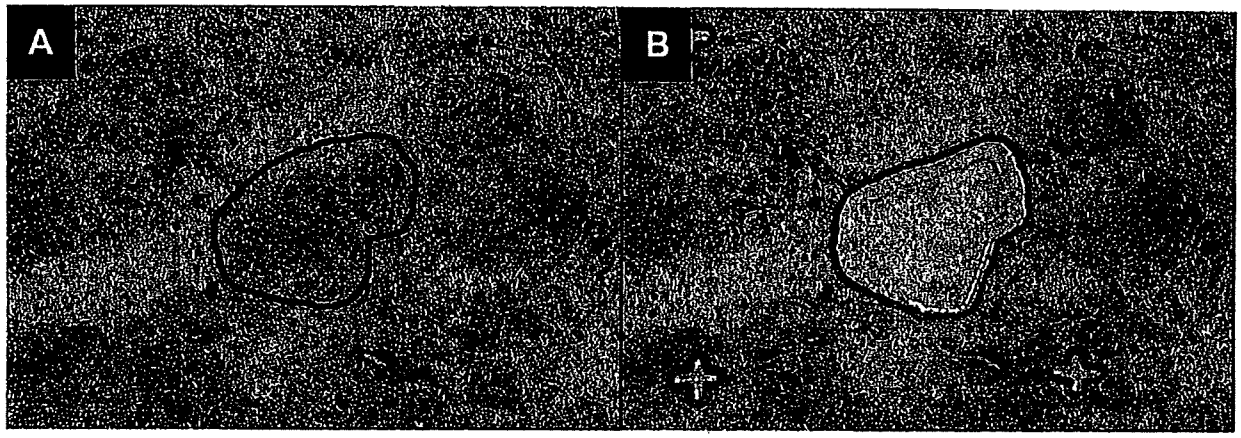


Figure 1. *Collection of giant cell using a laser microdissection (LMD) system. A: The giant cell tumor in the frozen section is stained with toluidine blue. B: Laser dissected section.*

The expression of tartrate-resistant acid phosphate (TRAP) was highest in the osteoclast-like giant cells (Figures 2-E and 3-B)

Discussion

The considerable level of BMP 2, 5 and 6 expression shown by the human GCT samples in comparison to the NOS-1 cells, indicated that such BMPs may contribute bone morphogenetic activity in human GCT. The relatively high level expression of BMP 2 in the cultured cells and the low level expression of BMP 2 in the osteoclast-like giant cells indicated that BMP 2 is expressed mainly in the stromal cells in GCT. The cultured tumor cells were analyzed after 3 to 5 passages because after three passages, the giant cells and monocytes were eliminated, leaving only stromal cells in the culture. Such stromal cells are thought to be the neoplastic element in GCT, and seem to originate from mesenchymal stem cells. Mesenchymal precursor cells exist in many different areas of the body, and differentiate to form mesenchymal progenitor cells (4-9). The expression of BMP 2 and 6 in the cultured cells from GCT in this study was consistent with previous studies which demonstrated considerable amounts of BMP 2 and 6 in the mesenchymal stem cells (31, 32). BMP 2 and 6 are thought to be the most potent agents for inducing osteoblastic lineage-specific differentiation in mesenchymal progenitor cells (10).

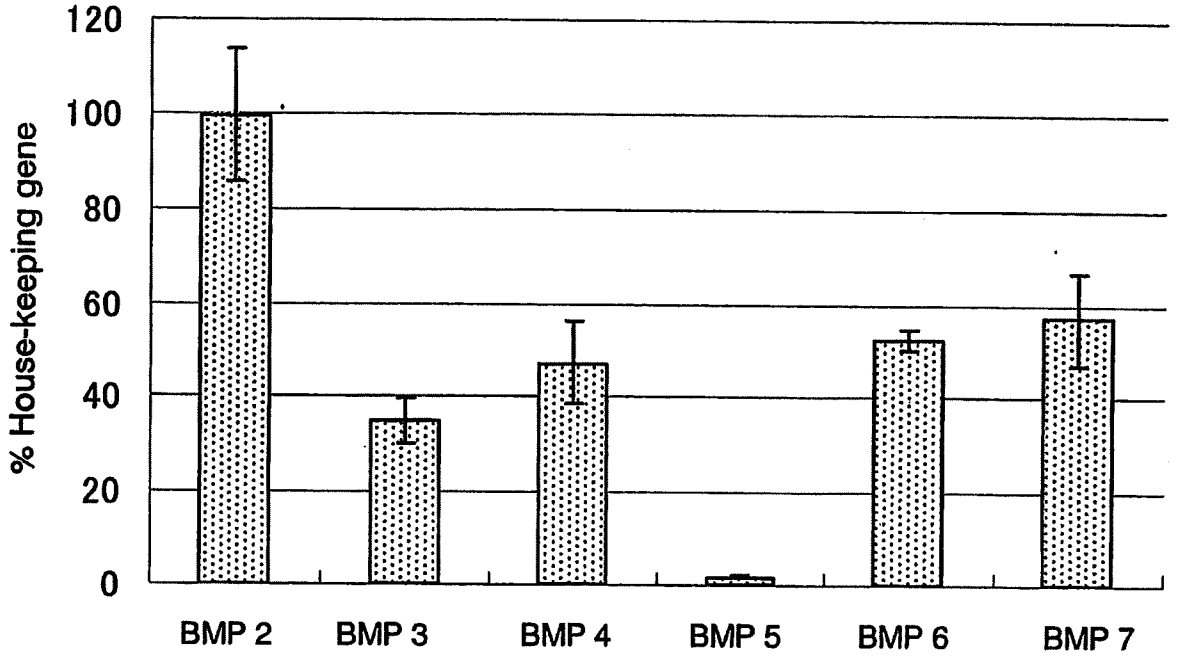
The relatively high level expression of BMP 5 in the LMD osteoclast-like giant cells and low level in the cultured cells indicated that BMP 5 is expressed mainly in the giant cells in GCT. A few reports regarding the expression of BMP 5, have indicated that it might play a fully paracrine role in rodent ovarian folliculogenesis, thereby regulating chondrocyte proliferation and differentiation (32-35). Cheng *et al.* showed that BMP 5 exhibited little osteogenic activity in osteoblastic progenitor cell lines, but it did stimulate alkaline phosphatase in relatively mature osteoblasts (10).

Table II. *Characteristics of the RNA samples.*

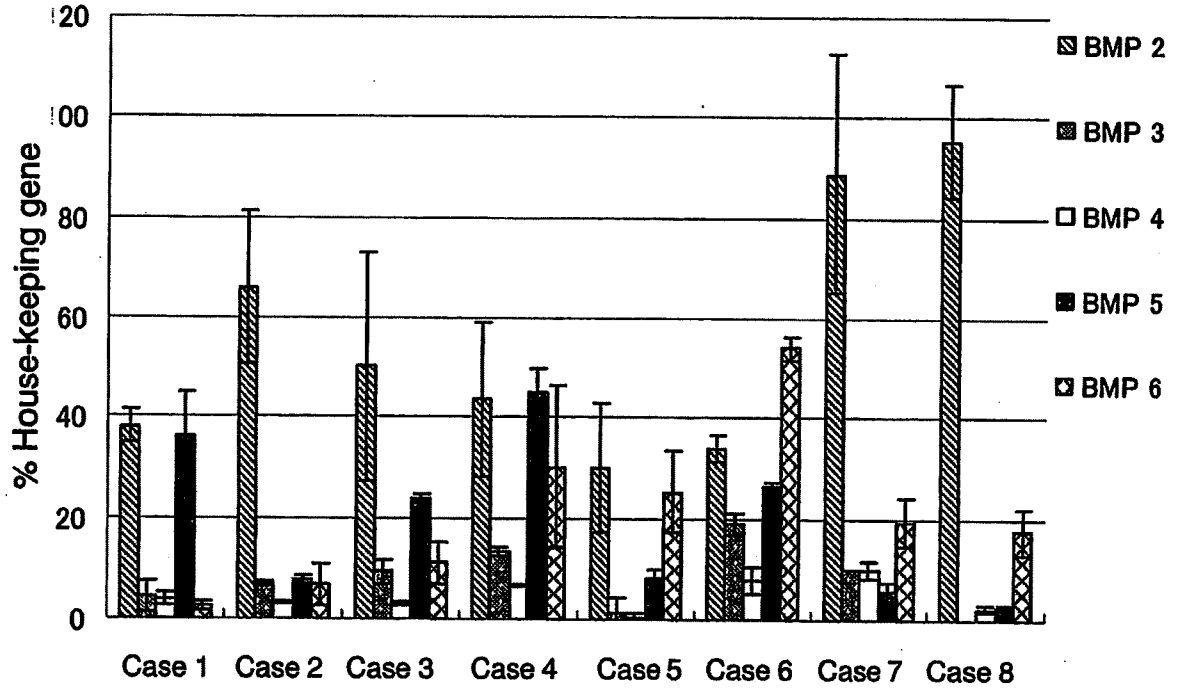
Case no.	Gender age (years)	Location		Tissue	RNA Cell	LMD
1	M 34	Lung	Metastasis	+1	-	-
2	M 39	Sacrum		+2	-	-
3	F 35	Radius		+3	+3*	-
4	F 46	Elbow	Soft tissue	+4	+4*	-
5	M 28	Fibula		+5	+5*	-
6	M 24	Fibula	Recurrence	+6	+6*	+6#
7	M 54	Lumbar spine	L1	+7	+7*	-
8	F 55	Femur		+8	+8*	+8**
9	F 31	Tibia		-	+9	-
10	M 36	Femur		-	-	+10

+: mRNA sample analysed, -: not analysed from GCT tissue, *cultured GCT cells, **LMD (laser microdissected) giant cells.

The osteoclast-like giant cells also showed considerable expression of BMP 6. Various immunohistochemical studies of BMP expression in osteoclasts have reprted that BMP 2, 4 and 7 were expressed in osteoclasts in a rat fracture model (19). BMP 7 was expressed in hamster osteoclasts (14), BMP 2, 3, 4, 5, 6 and 7 were expressed in the rat and human fetal growth plates (12). BMP 2, 4, 6 and 7 were expressed in mouse osteoclasts (18), and BMP 3 and 6 were expressed in human osteophytes, but BMP 2, 5 and 7 were not expressed (22). Because osteoclasts are potentially phagocytotic, it is possible that the BMPs present in the osteoclast cytoplasm may simply be the result of the phagocytosis of BMP-containing bone matrix. To date, there have been few reports of mRNA expression in osteoclasts.



A: Osteosarcoma cell line (NOS-1)



B: GCT tissue

Figure 2. *continued*

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Figure 2. *continued*

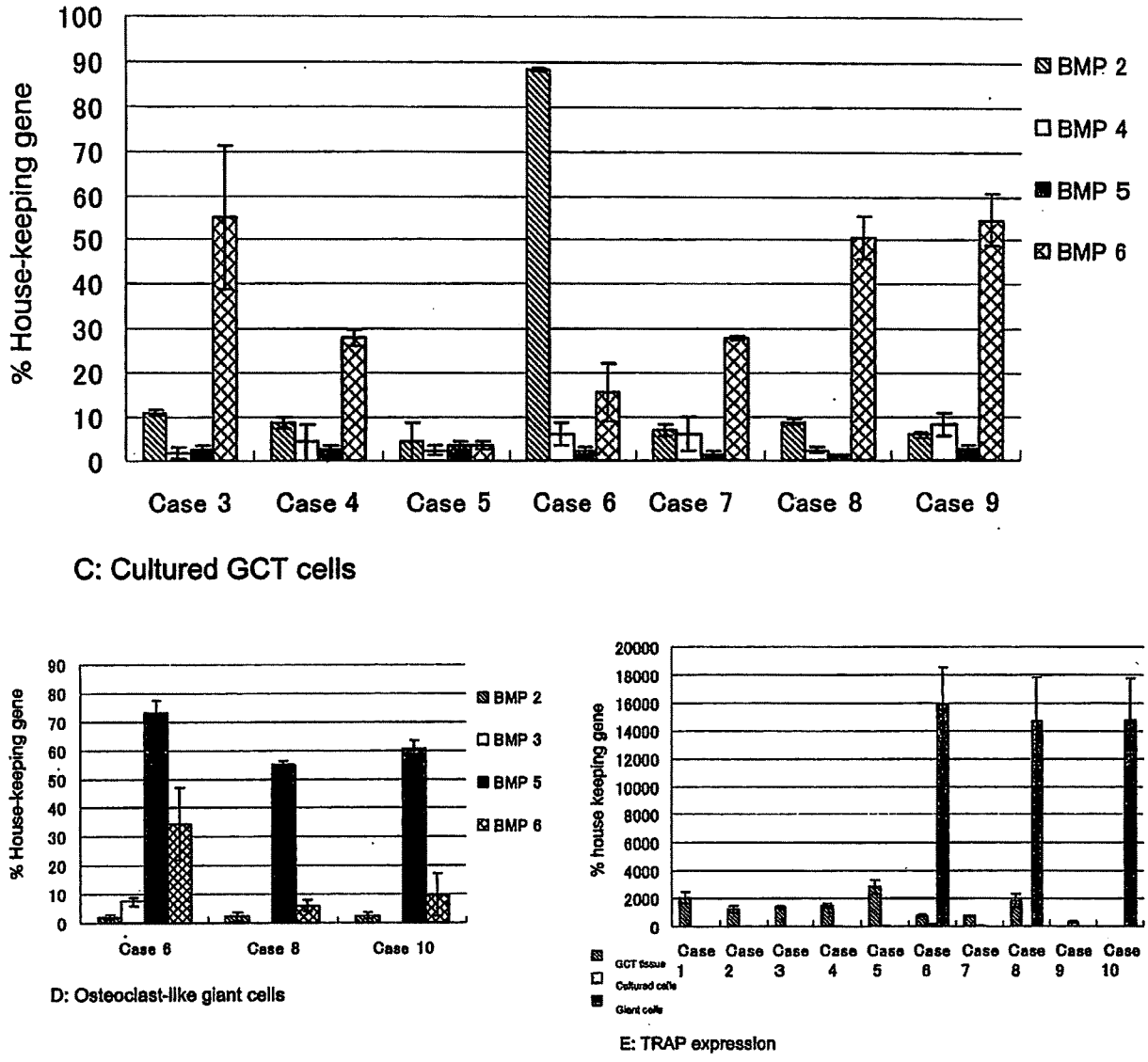

Figure 2. *BMP expression in osteosarcoma (NOS-1) cells (A), GCT tissue (B), cultured GCT cells (C), and osteoclast-like giant cells; (D) TRAP expression in GCT tissue, cultured cells and giant cells (E).*

TRAP is known as a specific marker for osteoclast (37, 38). The present study confirmed the overexpression of TRAP in the LMD osteoclast-like giant cells, which demonstrated the reliability of the LMD technique and the osteoclastic nature of the osteoclast-like giant cells in GCT, in line with the almost complete osteoclastic phenotype previously demonstrated in GCT giant cells (3-7, 36). Therefore, osteoclasts themselves probably produce various types of BMPs, including BMP 2, 3, 5 and 6.

There is ample evidence that bone formation is coupled to bone resorption (coupling phenomenon). The stimulation of bone resorption by agents such as prostaglandin E and parathyroid hormone is associated with increased bone formation. The mechanism whereby bone resorption facilitates bone formation is unknown. A local coupling factor linking bone resorption to subsequent bone formation may be the key regulator of the remodeling process. The bone matrix is a source of growth factors including BMPs, transforming growth

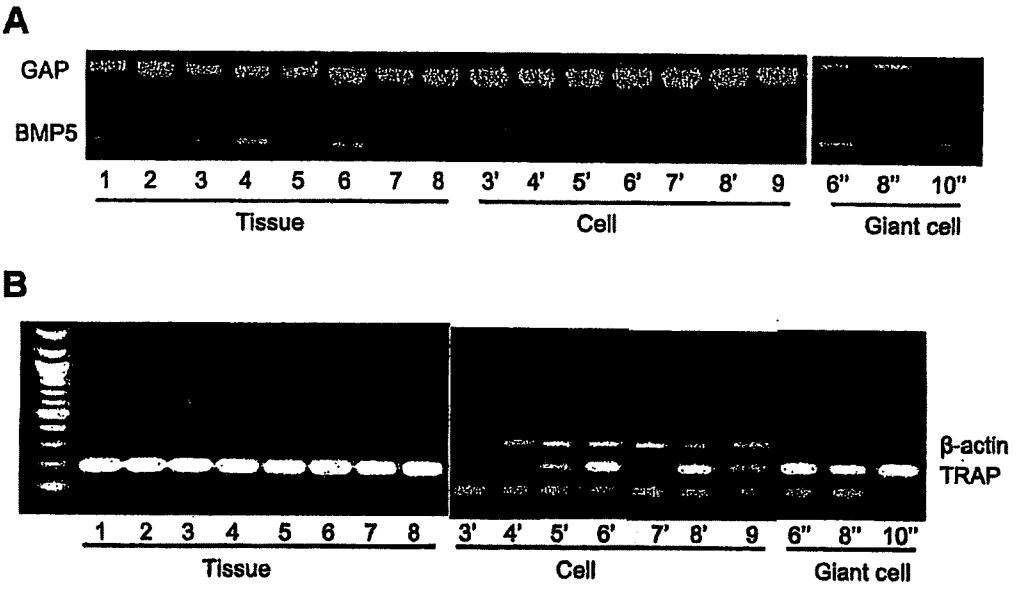

Figure 3. *RT-PCR of GCT tissue, GCT cultured cells and LMD giant cells. A: BMP 5 in comparison to GAPDH. B: TRAP in comparison to β-actin. The numbers refer to the samples in Table II.*

factors, insulin-like growth factors and fibroblast growth factors and it has been suggested that growth factors are released from the matrix during bone resorption by osteoclasts (37-39). In addition, a recent study indicated that osteoclast themselves could be the source of activity that contributes to the fine control that is a feature of the coupling phenomenon (38). A high level expression of BMP 5 and 6 in osteoclast-like giant cells in GCT may also support this hypothesis.

In conclusion, GCT tissue expresses BMP 2, 3, 4, 5 and 6 and cultured stromal cells express a high level of BMP 2 and 6. Purified LMD osteoclast-like giant cells also expressed BMP 5 and 6.

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Original article

Multiinstitutional phase II study of neoadjuvant chemotherapy for osteosarcoma (NECO study) in Japan: NECO-93J and NECO-95J

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Abstract

Background. Osteosarcoma is the most frequent primary malignant bone tumor. In Europe and the United States, its prognosis has been greatly improved by the use of multimodal treatment, including preoperative and postoperative chemotherapy as well as surgery. In Japan, however, only a few clinical studies on osteosarcoma have been carried out.

Methods. To evaluate the efficacy of neoadjuvant chemotherapy on nonmetastatic, operable osteosarcoma arising in the extremities, a prospective multiinstitutional phase II trial, the Neoadjuvant Chemotherapy for Osteosarcoma (NECO) study, was conducted. Preoperative chemotherapy included high-dose methotrexate (HD-MTX), cisplatin (CDDP), and adriamycin (ADR). If the induction therapy was assessed as not effective, high-dose ifosfamide (IFO) was added to the chemotherapy regimen. A total of 124 patients were enrolled in this trial, and ultimately 113 patients were eligible.

Results. The 5-year overall survival (OAS) and event-free survival (EFS) rates in the NECO study were 77.9% and 65.5%, respectively. A good histological response to the induction chemotherapy resulted in favorable OAS (78.7%). The patients assessed as poor histological responders with progressive disease after the induction chemotherapy exhibited comparable outcomes (OAS 89.5%, EFS 68.2%). There were no significant differences between the OAS and EFS rates of the patients in terms of response to preoperative chemotherapy.

Conclusions. We analyzed the results of the intensive neoadjuvant chemotherapy and the effects of adding IFO on patients with osteosarcoma in Japan. The results suggest efficacy of the high-dose IFO addition to the standard three-drug chemotherapy regimen. However, a randomized clinical study is needed to establish the true impact of IFO on patients with osteosarcoma.

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Introduction

Osteosarcoma, the most frequent primary malignant bone tumor, is characterized by the production of immature osteoid by tumor cells. The prognosis of osteosarcoma has been greatly improved by multimodal treatment, including preoperative and/or postoperative chemotherapy and surgery. Before the introduction of systemic chemotherapy, the long-term survival of patients treated with surgery alone was less than 20%; following introduction of systemic chemotherapy, the efficacy of adjuvant chemotherapy for osteosarcoma was suggested as the 5-year overall survival rose to 40%–60%.[1] The benefit of chemotherapy for osteosarcoma was then established by randomized clinical trials, which showed significantly improved survival among patients treated with adjuvant chemotherapy compared to those subjected to surgery alone.[2,3]

Recent studies have demonstrated a favorable outcome with the current therapy for patients with nonmetastatic operable osteosarcoma. The survival rates for patients treated with intensive multidrug chemotherapy and aggressive local control have been reported at 60%–80%.[4–10] Neoadjuvant preoperative chemotherapy failed to show any advantage over adjuvant chemotherapy regarding survival and limb-sparing rates.[4]

However, preoperative chemotherapy does have a benefit because the response to a neoadjuvant regimen is a strong prognostic factor for osteosarcoma patients. Survival rates for patients with 90% or more tumor necrosis in the surgical specimen were significantly higher than those with less tumor necrosis.[5,8,11]

The key drugs in the modern treatment of osteosarcoma include high-dose methotrexate (HD-MTX), cisplatin (CDDP), adriamycin (ADR), and/or ifosfamide (IFO). The addition of HD-MTX and/or IFO to CDDP + ADR failed to improve the outcome.[6,10,12–15]

Only a few clinical studies for patients with osteosarcoma have been carried out in Japan,[16–18] and none has been conducted by a multiinstitutional clinical study group to date. To evaluate the efficacy of preoperative neoadjuvant chemotherapy on nonmetastatic, operable osteosarcomas arising in the extremities in Japanese patients, we conducted a prospective multiinstitutional phase II trial, the Neoadjuvant Chemotherapy for Osteosarcoma (NECO) study. The trial consisted of two consecutive regimens: NECO-93J and its shortened counterpart NECO-95J. The preoperative chemotherapy in NECO regimen included HD-MTX, CDDP, and ADR. If the induction therapy was assessed as not effective, high-dose IFO (HD-IFO) was added to the chemotherapy regimen. We analyzed the results of the intensive neoadjuvant chemotherapy and the effects of adding IFO.

Materials and methods

Patients

A total of 124 patients with osteosarcoma were enrolled in this study between October 1993 and May 2001. Eligibility criteria for inclusion in the study were (1) a histologically proven high-grade osteosarcoma found in a biopsy specimen; (2) a localized, measurable tumor in the extremities; (3) an operable tumor; (4) no evidence of distant metastasis, evaluated by computed tomography (CT) and bone scintigraphy; (5) no history of treatment for the sarcoma; (6) age ≤30 years; (7) an Eastern Cooperative Oncology Group (ECOG) performance status of 0–3; (8) sufficient organ function, i.e., creatinine clearance ≥75 ml/min, serum uric acid ≤7.8 mg/dl, serum glutamic oxalic transaminase (SGOT) and serum glutamic pyruvic transaminase (SGPT) that were less than twice the normal upper limit, WBC ≥ 4000/mm^3, hemoglobin ≥10 g/dl, and platelets ≥100 000/mm^3; (9) informed consent had been obtained. Secondary osteosarcoma, periosteal osteosarcoma, parosteal osteosarcoma, and low-grade osteosarcoma were excluded from this study. The radiological evaluation of the tumor must have been carried out prior to entry. The evaluation included conventional radiography, magnetic resonance imaging (MRI), and/or CT.

Among 124 osteosarcoma patients, 6 with metastasis, 2 with age >30 years, 2 with the tumor having arisen in the trunk, and 1 with insufficient organ function were not included in this analysis. The remaining 113 patients with untreated high-grade osteosarcoma arising in the extremities were analyzed.

The participating institutions (from north to south) and the number of eligible patients recruited from each institute in the NECO study are as follows: Sapporo Medical University 5, Hokkaido Cancer Center 21, Chiba Cancer Center 16, National Cancer Center Hospital 19, Teikyo University 3, Cancer Institute Hospital 2, Kanagawa Cancer Center 8, Yonago Medical Center 2, Tottori University 2, Okayama University 20, Kyushu University 15. This study did not receive institutional review board approval because it was carried out from 1993 to 2001. The participants in the NECO trial did not declare any conflicts of interest.

Chemotherapy regimen for NECO-93J

Preoperative neoadjuvant chemotherapy

The patients received induction chemotherapy consisting of two courses of high-dose methotrexate (HD-MTX) (12 g/m^2 for patients <15 years of age and 8–10 g/m^2 for those ≥15 years) followed by a course of cisplatin (CDDP) (120 mg/m^2) and adriamycin (ADR) (30 mg/m^2/day × 2 days) (phase I) (Fig. 1). HD-MTX was administrated at 6 h; and 24 h after the initiation of HD-MTX, leucovorin was given at a dose of 15 mg every 6 h for 10 doses.

After the induction chemotherapy, the patients were assessed for their response using MRI, CT, and/or plain radiography of the primary tumor. For the patients whose tumors were assessed as stable disease (SD), partial response (PR), or complete response (CR), defined as below, four courses of HD-MTX and one course of CDDP + ADR were given (phase II). If the clinical response was assessed as progressive disease (PD), the preoperative chemotherapy was changed to two courses of high-dose ifosfamide (HD-IFO) (total 16 g/m^2; 4 g/m^2 on the first day followed by 2 g/m^2/day × 6 days) combined with mesna at the same dose as IFO (4 g/m^2 × 1 day and 2 g/m^2/day × 6 days) (phase III). The chemotherapy regimens were carried out with a 3-week interval if the creatinine clearance was ≥75 ml/min, serum uric acid ≤7.8 mg/dl, SGPT ≤ twice the normal upper limit, WBC ≥ 4000/mm^3, hemoglobin ≥10 g/dl, and platelets ≥100 000/mm^3.

The primary osteosarcoma was resected after the preoperative chemotherapy described above. The resected tumor was assessed histologically for tumor necrosis.