In addition, apoptosis signaling is also regulated by chaperones; SODD (silencer of death domains) interacts with the DD (death domain) of death receptors such as Fas, TNF-αR, and prevents binding molecules which are necessary for activation of caspases or the transcription factor NF-κB. Soon after the receptor is triggered, SODD relocates to the receptor, and leads to the termination of the death signal(13).

Inside the cells, apoptosis can be also regulated at different levels. Two major proteins have been identified to modulate apoptosis (Figure 3). The members of Bcl-2 family constitute a first class of regulatory proteins, which act at the mitochondrial level. The Bcl-2 family is characterized by Bcl-2 homology (BH) domains, BH1-4. According to their functions, they are categorized to anti-apoptotic (e.g. Bcl-2, Bcl-xL) and pro-apoptotic proteins (e.g. Bax, Bak) (14). The anti-apoptotic group is characterized by BH4 domain, whereas BH3 domain is pivotal for apoptosis induction. This is illustrated by a subgroup of the pro-apoptotic Bcl-2 family members, the BH3 domain-only proteins (e.g. Bid, Bad, Bim). It is suggested that the ratio between pro- and anti-

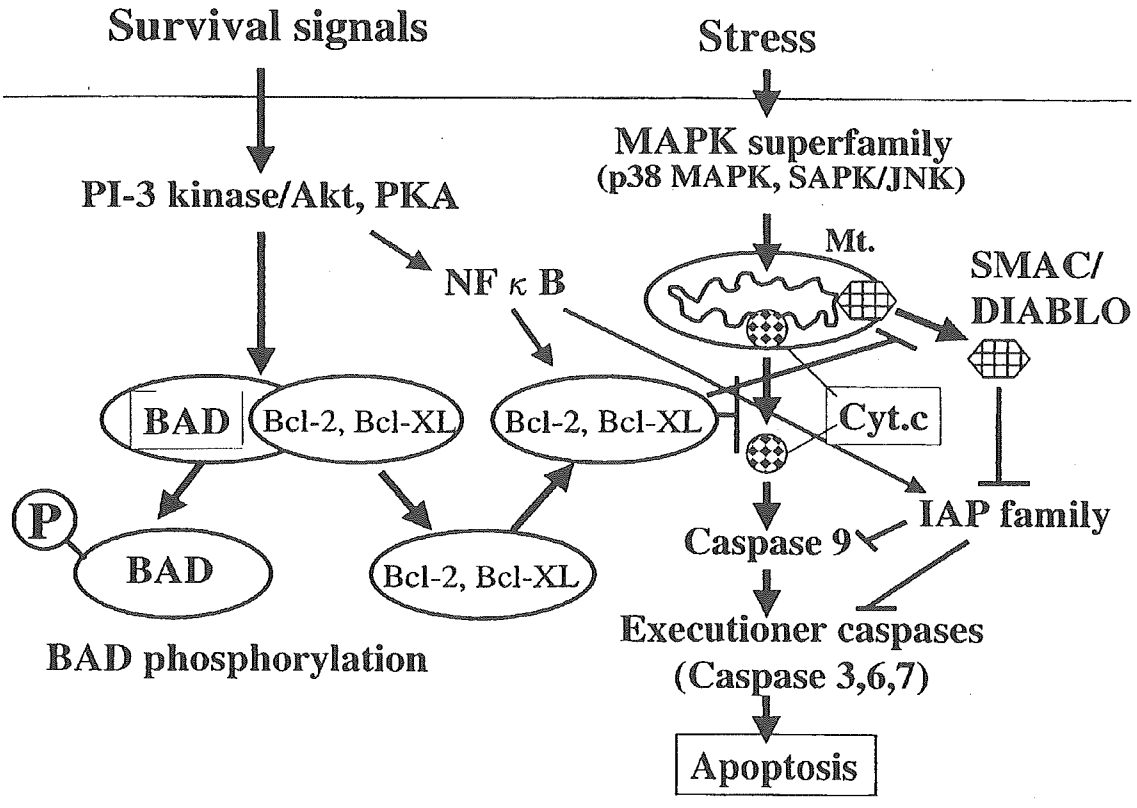

Figure 3. Regulation of mitochondrial pathway (intrinsic pathway). The members of Bcl-2 family regulate apoptosis, especially mitochondrial pathway. Activation of mitochondria by various stress leads to the release of cytochrome c (Cyt.c) into the cytosol. In turn, caspase 9 is activated, leading to apoptosis. Mitochondrial pathway is inhibited by anti-apoptotic proteins including Bcl-2, Bcl-XL, IAPs which are regulated by SMAC/DIABLO. Survival signals phosphorylate and inactivate pro-apoptotic member BAD. Consequently, Bcl-2 or Bcl-XL restores its anti-apoptotic action. In addition, survival signals induce IAPs through NF-κB activation. Mt., mitochondria; SMAC/DIABLO, second mitochondria-derived activator of caspase/direct IAP binding protein with low pI.

apoptotic family members should determine the cells' fate. Furthermore, it seems to get more complex with the involvement of mitochondrira in the apoptotic pathway. A prominent feature of the Bcl-2 family is the ability to interact with one another to form heterodimers, and in some cases, homodimers. BAD exerts its pro-apoptotic action on the mitochondria in the non-phosphorylated state, and forms inactivating dimmers with Bcl-2 or Bcl-XL to promote apoptosis (15, 16). Phosphorylated BAD dissociates from the heterodimeric complex with Bcl-2 or Bcl-XL, restoring Bcl-2 or Bcl-XL function, and activating cell survival signaling. Recent works have indicated that activated BAD kinases including Akt or PKA(A kinase) in the tumor cells play a key role in apoptosis resistance through BAD phosphorylation (17, 18). In this way, survival signals including Akt and PKA regulate apoptotic signaling through phosphorylation of BAD.

Furthemore, the IAPs (inhibitor of apoptosis proteins) constitutes a second class of regulatory proteins (19). IAPs bind and inhibit caspases. There have been nine IAPs identified in human cells including X-IAP (hILP, MIHA, ILP-1), c-IAP1 (MIHB, HIAP-2), c-IAP2 (HIAP-1, MIHC, API2), NAIP, ML-IAP, ILP2, livin (KIAP), apollon and survivin. These IAPs have been characterized by baculoviral IAP repeat (BIR). IAPs are prevented by other molecule termed SMAC/DIABLO (second mitochondria-derived activator of caspase /direct IAP binding protein with low pI), which is released along with cytochrome c and promotes caspase activity by binding and inhibiting IAPs. It has been described that expression of c-IAP2 is regulated by transcriptional factor NFκB(20) and that NF-κB is a target gene of survival signals (21). Taking together, survival signals regulate apoptotic signaling also through expression of IAP family under NF-κB control.

II. Apoptosis induction in the tumor cells

a) Induction by immune system

The major effector cells against tumors are cytotoxic T lymphocytes (CTL) of the adaptive immune system, and natural killer (NK) cells of innate immune system.

CTLs and NK cells use two major mechanisms to guide apoptosis: the FasL (CD95L) pathway which is calcium-independent, and granule exocytosis pathway which is calcium-dependent. In the former pathway, CTL exhibits FasL on the cell surface and triggers apoptosis through the binding of FasL to Fas on the cell surface of the tumor cell (22).

In contrast, CTLs and NK cells secrete a membrane permeability protein called perforin and proteolytic enzyme, known as granzyme, towards the target tumor cell. Perforin can penetrate the cell membrane and allow granzyme into the target cell in calcium-dependent manner. In turn, granzyme, a serine protease, activates capsase cascade (23). IFN γ secretion from NK cells is another mechanism of induction of apoptosis.

Resistance of the tumor cells to these mechanisms inducing apoptosis, not only leads to the escape from immune surveillance but also influences the efficacy of immuno-therapy.

b) Therapeutic induction

Stresses such as chemotherapy and γ-irradiation can initiate apoptosis of the tumor cells primarily, and failure to activate the apoptotic program generates drug resistance in

tumor cells (24). A key element in stress-induced apoptosis is p53 protein. The transcriptional activity of p53 protein is crucial for its pro-apoptotic function; p53 protein can induce proteins involved in mitochondrial pathway including BAX, NOXA, and also in death receptor pathway such as Fas, TRAIL-R1, and TRAIL-R2 (25, 26). Other stress pathways which are activated in response to chemotherapy, are stress-activated protein kinase (SAPK) / Jun kinase (JNK) or p38 MAPK, and these both consist of MAPK superfamily. SAPK can regulate activity of AP-1 transcriptional factors, leading to induction of pro-apoptotic proteins including FasL and TNFα.

Principally, Fas/FasL system plays an essential role in anti-cancer drug-induced apoptosis. Anti-cancer drugs induce Fas in p53-dependent manner, and also up-regulate FasL through SAPK activation (27). Eventually, up-regulation of Fas and FasL may allow the cells to commit suicide or kill neighboring cells.

In any case, the apoptotic pathways depend on the stress stimulus, the cell type and the tumor environments.

III. Apoptosis resistance in tumor cells

Resistance of tumor cells to apoptosis brings the limited efficiency of immune- and drug- induced destruction of tumors. Tumor cells can acquire apoptosis resistance by a wide range of mechanisms that interfere with apoptotic signaling at different levels (Figure 4).

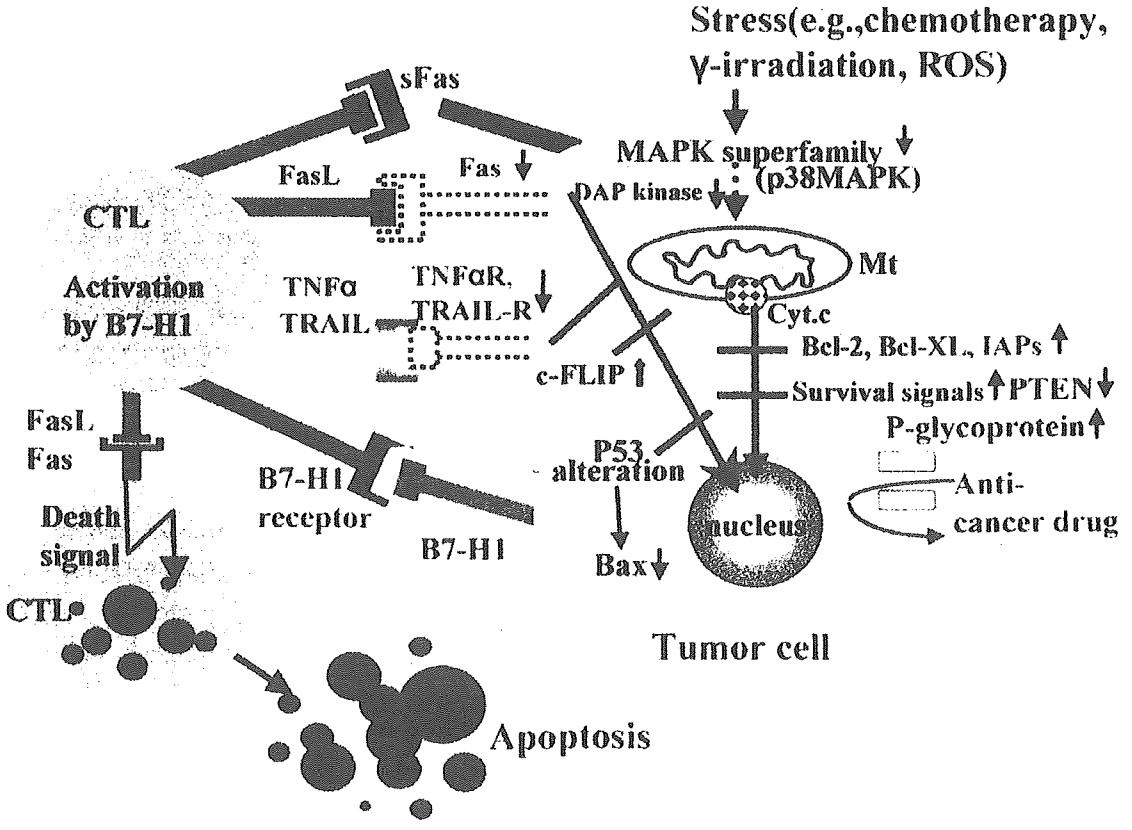

Figure 4. Apoptosis resistance in tumor cell. Tumor cells can acquire apoptosis resistance by a wide range of mechanisms that interfere with apoptotic signaling at different levels.

a) Down regulation of pro-apoptotic signaling

Alteration of pro-apoptotic molecules results in down-regulation of apoptotic signaling; death receptors are down-regulated or inactivated in a variety of tumors including HCC(hepatocellular carcinoma) or colon cancer compared with their normal counter-parts. Expression of Fas is down-regulated in HCCs with poor differentiation, which provides a prognostic significance for disease-free survival (28). It has been reported that soluble Fas (sFas), generated by alternative splicing of the Fas gene, can block apoptosis through pre-receptor binding with FasL. In this context, the serum level of sFas is elevated in the patients with HCCs, which suggests that sFas may cause apoptosis resistance (29).

In addition, TRAIL-R1, R2 down-regulation is identified in HCCs, which may attenuate anti-tumor effect of TRAIL (30). In contrast, it has been shown that hepatitis B virus X protein up-regulates expression of TRAIL-R1, leading to enhancement of TRAIL-inducing apoptosis in liver cancer cells (31).

In human colon cancer cell line, DISC formation is deficient. TRAIL-R1 or caspase 8 is not recruited to the DISC, leading to resistance to TRAIL-mediated apoptosis (32). On the other hand, death-associated protein-kinase (DAP-kinase) was identified as a positive mediator of apoptosis induced by interferon γ, and expression of DAP-kinase is lost or reduced in human HCCs, which is inversely correlated with disease-free survival rate or overall survival rate (33).

As it is well known that p53 protein plays a fundamental role in apoptosis in terms of inducing Fas, TRAIL-R1, TRAIL-R2, Bax or NOXA, alterations of p53 protein influence the sensitivity to apoptosis. Indeed, p53 gene mutation occurs preferentially in moderately and poorly differentiated HCCs as a late event of HCC progression (34), which may account, in part, for loss of Fas expression in HCCs (35). Furthermore, without any mutation on the p53 gene, viral products can modulate p53 function *in vitro*, accompanied by apoptosis resistance. For instance, hepatitis B virus X protein complexes with p53 protein in the cytoplasm, and prevents its nuclear entry and ability to induce apoptosis (36).

On the other hand, down-regulation of Bid, a pro-apoptotic member of Bcl-2 family, has been demonstrated to be involved in apoptosis resistance. Hepatitis B virus X protein induces down-regulation of Bid *in vitro*, and expression of Bid was significantly lower in human HCCs compared to that of their corresponding non-cancerous tissues (37), and which suggests that reduction of Bid expression may generate the development of human HCCs.

It remains, so far, controversial whether products of hepatitis virus may enhance or attenuate apoptotic signaling; it depends on the target molecule and/or conditions applied.

Alteration of apoptosis-inducing signaling in the tumor cells may be as another possible explanation for apoptosis resistance. For example, p38 MAPK pathway is attenuated in HCCs compared with non-cancerous counterparts of the liver, and activation of p38MAPK can induce apoptosis in human hepatoma cells (38). These findings indicate that down-regulation of p38 MAPK may be concerned with apoptosis resistance.

Furthermore, over-expression of COX-2 inhibits TRAIL-R2 expression, leading to resistance to TRAIL in colon cancer cells (39). Moreover, neoplastic transformation of human colon epithelium leads to a loss of Fas expression on cell surface giving on

account of either to a stable lack of Fas or to an INF-γ/TNFα sensitive phenotype of inducible Fas expression (40).

b) Up-regulation of anti-apoptotic signaling

Up-regulation of Bcl-2 family confers resistance to multiple apoptosis-inducing pathways in the tumor cells, including gastric cancers and colon cancers. For example, Bcl-2 mRNA level is significantly increased in gastric adenocarcinoma with *H. pylori.* infection (41). Moreover, Bcl-2 protein seems to be connected with colorectal tumorigenesis in the early phase of adenoma-carcinoma sequence (42).

Another anti-apoptotic member of Bcl-2 family, Bcl-XL has been described to be up-regulated and involved in apoptosis resistance of HCCs (43, 44). On the other hand, increased expression of c-FLIP(cellular FLIP) has been also described in human HCCs and colon carcinomas (45, 46). In addition, it has been shown that tumors with up-regulated c-FLIP can escape from T-cell mediated immunity (47). In this way, elevated c-FLIP seems to trigger apoptosis resistance, accompanied by immune escape.

IAP family also seems to be involved in apoptosis resistance in HCCs or the tumors in gastroiutestinal tract. For instance, survivin, one of the IAP family, is up-regulated in HCCs, which is predictive of recurrence of disease (48, 49). Besides, survivin is up-regulated in gastric cancer (50) and UC (ulcerative colitis)-associated adenocarcinoma of the colon (51). Expression of the X-linked inhibitor of apoptosis(X-IAP) is enhanced in HCCs and liver cancer cell lines (52). Moreover, another IAP family member, c-IAP2, is affected by the translocation t (11; 18)(q21;q21) that is found in approximately 50% of MALT lyomphomas, which results in development of the tumor (53).

c) Augmentation of survival signals

Constitutively active survival signaling partly sets up apoptosis resistance in many kinds of tumors. For example, alteration of PTEN (Phosphatase and Tensin homolog deleted on chromosome Ten) may provide a possible explanation for activated survival signaling in the tumors. Alleic loss or LOH of chromosome 10q has been frequently characterized in HCC, implying the presence of tumor suppressor gene on it (54). In this regard, tumor suppressor gene PTEN has been identified on chromosome 10q23 (55), and PTEN protein encodes dual-specificity protein phosphatases. The two major substrates for PTEN are phosphatidylinositol triphosphate (PIP3) phospholipides and the oncogene Akt/PKB, both of which play crucial roles in regulating survival signals of the cells. Thus, functional alteration of PTEN through a variety of mechanisms, such as somatic mutations, hemizygous deletions, and promoter methylation, can enhance survival signals (56). Loss of a PTEN allele, for instance, has been described in 20-30% of HCC patients (57), and reduced protein expression of PTEN was correlated with increased tumor grade, or advanced disease stage (58). Taking together, functional alteration of PTEN in HCCs may lead to an activated survival signaling that enhances apoptosis resistance.

On the other hand, as described above, BAD exerts its pro-apoptotic action in non-phosphorylated form, and forms inactivating dimmers with anti-apoptotic Bcl-2 family, Bcl-2 or Bcl-XL. Once Bad is phosphorylated, it dissociates from the complex. Consequently, Bcl-2 or Bcl-XL restores its anti-apoptotic action and prevents apoptosis. In this regard, BAD phosphorylation seems to be a key event to determine the cells' fate.

Recent report has revealed that MAPK (mitogen-activated protein kinase) is one of the BAD kinases (59). In this context, MAPK activation has been described in human HCCs (60). In addition, over-expression of human IRS-1(insulin receptor substrate-1) protein, which is often observed in HCCs, induces not only activation of PI-3 kinase/Akt pathway (61) but also MAPK activation (62). Consequently, activated MAPK and Akt pathways in HCCs seem to induce apoptosis resistance through BAD phosphorylation.

Moreover, products of hepatitis viruses seem to modulate survival signaling in HCCs. Hepatitis B virus X protein or hepatitis C virus NS5A protein activates PI-3 kinase/Akt dependent survival signaling *in vitro* (63, 64).

d) Multi-drug resistance

Multi-drug resistance is a major factor in the failure of most forms of chemotherapy. Tumors basically consist of mixed population of tumor cells. Some of those are drug-sensitive, while others are drug-resistant. Chemotherapy kills drug-sensitive cells, but leaves behind a higher population of drug-resistant cells.

Ergo, much as chemotherapy on tumors is substantially curative, its effectiveness is limited. Moreover, some tumor cells, which are initially sensitive, often acquire resistance not only to the initial therapy, but also to other drugs which have not been applied (65).

Resistance to chemotherapy can be attributed to the presence of a molecular transporter which expels chemotherapeutic agents from the tumor cells. The two transporters have been identified to confer resistance to chemotherapy; *MDR1* gene product P-glycoprotein and MRP (multi-drug resistance protein) (66).

It has been reported that P-glycoprotein is over-expressed in various kinds of tumors including gastrointestinal cancer (67) as well as the tumors that acquire drug resistance after chemotherapy, and it confers resistance to a variety of structurally and functionally un-related anti-cancer drugs.

MDR1 promoter activity is up-regulated by a wide range of stimuli, such as anti-cancer drugs, DNA-damaging agents, heat shock, and UV irradiation, which may be involved in acquired drug-resistance to the initial therapy with anti-cancer drugs (68). *MDR1*gene expression can be also up-regulated as a consequence of mutation of tumor suppressor gene *p53*or activated *ras* oncogene.

Moreover, insulin-like growth factor-1 promotes multidrug resistance in colon cancer cells partly through *MDR1* gene up-regulation (69).This evidence indicates that survival signaling activated by IGF-1 may invite drug resistance of tumor cells to anticancer drugs.

In addition to effluxing drugs, P-glycoprotein may play a specific role in regulating caspase-dependent apoptotic signaling by FasL, TNFα, UV irradiation (70). Thus, it can be speculated that over-expression of P-glycoprotein in the tumor cells may also alter intrinsic or extrinsic apoptotic signaling, leading to apoptosis resistance.

Since P-glycoprotein seems to play a role in intrinsic and acquired multi-drug resistance, many drugs have been applied in order to modulate function of P-glycoprotein and reverse multidrug resistance. In this context, it has been reported that reversing agents, such as verapamil, cyclosporin A, diazepam, are substrates for P-glycoprotein-mediated transport and competitively prevent P-glycoprotein transport of anti-cancer drugs (71). A new generation of the multidrug resistance reversing agents

has been developed, and is being applied in clinical trials. However, since P-glycoprotein is expressed in normal tissues such as liver, kidney, jejunum, colon and adrenal gland (72), P-glycoprotein inhibitors basically exert deleterious effects on normal tissues, and which needs to be carefully evaluated.

On the other hand, MRP expression has been described in several kinds of tumors including colon cancers, *in vivo* and *in vitro* (73).

Another possible mechanism for resistance to chemotherapy is attributed to an imbalance between pro-apoptotic signaling and anti-apoptotic (survival) signaling in the tumor cells. Because a series of chemotherapeutic agents induce tumor cell death mainly through activating apoptotic signaling, alteration of apoptotic signaling described above, is related to multi-drug resistance. Since survival signals are up-regulated by a number of mechanisms in the tumor cells, survival signaling can be one of the targets to reverse multi-drug resistance. In this context, Hsp90 (heat shock protein) has been described to bind with not only Akt but also with PDK1, an upstream Akt kinase, and regulate survival signaling (74, 75). Therefore, Hsp90 may be a promising target in order to suppress PDK1-Akt- mediated survival signaling pathway.

PDK1 has been proposed to be another molecular target to regulate survival signaling; staurosporin analogue and topotecan inhibit activity of PDK1 *in vitro*, resulting in down –regulation of survival signaling in the tumor cells (76, 77).

These findings indicate that molecules involved in survival signaling may be a promising target to develop a new therapeutic strategy of chemotherapy.

e) Counterattack by tumor cells

Immune evasion can participate in apoptosis resistance of the tumor cells.
The counterattack hypothesis that cancer cells express FasL on the cell surface and are able to counter attack Fas-expressing tumor-infiltrating activated T cell, has been proposed as one of the possible explanations for immune evasion (78). However, a recent report denies the ability of FasL-expressing colon cancer cells to induce apoptosis in Fas-sensitive target cells *in vitro* (79). On the other hand, there is an evidence that instead of being killed by tumor cells tumor-infiltrating cytotoxic T lymphocytes express FasL and undergo Fas-dependent cell death in an auto- or paracrine manner following MHC-restricted recognition of tumor antigens (80). Furthermore, B7-H1, a recently described member of the B7 family of co-stimulatory molecule, has been shown to be up-regulated on the cell surface of tumor cells including lung cancer, ovarian cancer, and colon cancer. And B7-H1 drives activated tumor-reactive T-cells into apoptosis (81). In this context, binding of B7-H1 with the corresponding receptor(s) on T cells induces release of IL-10 from T cells, and also up-regulates expression of Fas and FasL on the cell surface of T cells. Consequently, tumor-associated B7-H1 promotes T cell apoptosis. In conjunction with cancer vaccine or adoptive immunotherapy, selective manipulation of B7-H1 pathway needs to be shed light on.

Conclusion

Since the molecular mechanisms of apoptosis induction have been revealed in the last decade, many therapeutic strategies have been proposed against apoptosis resistance in the tumor cells. However, a macroscopic tumor tissue is heterogeneous and it is likely that different cells in the same tumor tissue have different mechanisms of apoptosis

resistance. In addition, multiple mechanisms for apoptosis resistance have developed in a single tumor cell. Therefore, combination of therapeutic strategies is required to circumvent apoptosis resistance. Moreover, targeting of the therapy is needed not to disturb normal cell growth. The modulation of apoptosis and its therapeutic application would perform a major role in the future flied of clinic.

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References

1. Vaux, D.L., & Korsmeyer, S.J. 1999, *Cell* 96, 245.
2. Krammer, P.H. 1999, *Adv. Immunol.*71, 163.
3. Nicholson D.W. 1999, *Cell Death Differ.* 6, 1028.
4. Shmitz, I., Kirchhoff, S., & Krammer, P.H. 2000, *Int. J. Biochem. Cell. Biol.* 32, 1123.
5. Nakagawa, T., Zhu, H., Morishima, N., Li, E., Xu, J., Yanker, B.A., & Yuan, J. 2000, *Nature* 403, 98.
6. Ashkenazi, A., & Dixit, V.M. 1998, *Science* 281, 1305.
7. Scaffidi, C., Fulda, S., Srinivasan, A., Friesen, C., Li, F., Tomaselli, K.J., Debatin, K.M., Krammer, P.H., & Peter, M.E. 1998, *EMBO J.* 17, 1675.
8. Goldstein, J.C., Waterhouse, N.J, Juin, P., Evan, G.I., & Green, D.R. 2000, *Nature Cell Biol.* 2,156.
9. Rathmell, J.C., & Thompson, C.B. 1999, *Annu. Rev. Immunol.* 17,781.
10. Sperandio, S., De Belle, I., & Bredesen, D.E. 2000, *Proc. Natl. Acd. Sci. U.S.A.* 97, 14376.
11. Peter, M.E., Hellbardt, S., Schwartz-Albiez, A., Westendorp, M.O., Walczak, H., Moldenhauer, G., Grell, M., & Krammer, P.H. 1995, *Cell Death Diff.* 2,163.
12. Krueger, A., Baumann, S., Krammer, P.H., & Kirchhoff, S. 2001, *Mol. Cell. Biol.* 21, 8247.
13. Jiang, Y., Woronicz, J.D., Liu, W., & Goeddel, D.V. 1999, *Science* 283, 543.
14. Kroemer, G. 1997, *Nat. Med.* 3,614.
15. Gross, A., McDonnell, J.M., & Korsmeyer, S.J. 1999, *Genes.Dev.* 13,1899.
16. Kekekar, A., Chang, B.S., Harlan, J.E., Fesik, S.W., & Thompson, C.B. 1997, *Mol.Cell.Biol.* 17, 7040.
17. Datta, S.R., Dudek, H., Tao, X., Masteres, S., Fu, H., Gotoh, Y., & Greenberg, M.E. 1997, *Cell* 91, 231.
18. Harada, H., Becknell, B., Wilm, M., Mann, M., Huang, L.J., Taylor, S.S., Scott, J.D., & Korsmeyer, S.J. 1999, *Mol. Cell.* 3, 413.
19. Deveraux, Q.L., & Reed, J.C. 1999, *Genes Dev.* 13, 239.
20. Chu, Z-L., McKinsey, T.A., Liu, L., Gentry, J.J. Malim, M.H., & Ballard, D.W. 1997, *Proc. Natl. Acad. Sci. U.S.A.* 94, 10057.
21. Romashkova, J.A., & Makarov, S.S. 1999, *Nature* 402, 86.
22. Shresta, S., Pham, C.T., Thomas, D.A., Graubert, T.A., & Ley, T. 1998, *Curr. Opin. Immunol.* 10, 581.
23. Heusel J.W., Wesseischmidt, R.L., Shresta, S., Russell, J.H., & Ley, T.J. 1994, *Cell* 76, 977.
24. Herr, I., & Debatin, K. M. 2001, *Blood* 98, 2603.
25. Miyashita, T., & Reed, J.C. 1995, *Cell* 80, 293.
26. Muller, M, Wilder, S., Bannasch, D., Israeli, D., Lehlbach, K., Li-Weber, M., Friedman, S.L., Galle, P.R., Stremmel, W., Oren, M., & Krammer, P.H. 1998, *J. Exp. Med.* 188, 2033.
27. Muller, M., Strand, S., Hug, H., Heinemann, E.M., Walczak, H., Hofmann, W.J., Stremmel, W., Krammer, P.H., & Galle, P.R. 1997, *J. Clin. Invest.* 99, 403.
28. Ito, Y., Monden, M., Takeda, T., Eguchui, H., Umeshita, K., Nagano, H., Nakamori, S., Dono, K., Sakon, M., Nakamura, M., Tsujimoto, M., Nakahara, M., Nakao, K., Yokosaki, Y., & Matsuura, N. 2000, *Br. J. Cancer* 82, 1211.

29. Lee, S.H., Shin, M.S., Lee, H.S., Bae, J.H., Lee, H.K., Kim, H.S., Kim, S.Y., Jang, J.J., Joo, M., Kang, Y.K., Park, W.S., Park, J.Y., Oh, R.R., Han, S.Y., Lee, J.H., Kim, S.H., Lee, J.Y., & Yoo, N.J. 2001, *Hum. Pathol.* 32, 250.
30. Shin, E.C., Seong, Y.R., Kim, C.H., Kim, H., Ahn, Y.S., Kim, K., Kim, S.J., Hong, S.S., & Park, J.H. 2002, *Exp. Mol. Med.* 34, 114.
31. Janssen, H.L., Higuchi, H., Abdulkarim, A., & Gores, G.J. 2003, *J. Hepatol.* 39, 414.
32. Jin, Z., McDonald, E.R., 3rd, Dicker, D.T., & El-Deiry, W.S. 2004, *J. Biol. Chem.* 279, 35829.
33. Matsumoto H, Nagao, M, Ogawa S, Kanehiro, H., Hisanaga, M., Ko, S., Ikeda, N., Fujii, H., Koyama, F., Mukogawa, T., & Nakajima, Y. 2003, *Anticancer Res.* 23, 1333.
34. Oda, T., Tsuda, H., Scarpa, A., Sakamoto, M., & Hirohashi, S. 1992, *Cancer Res.* 52, 6358.
35. Volkmann, M., Schiff, JH, Hajjar, Y., Otto, G., Stilgenbauer, F., Fiehen W., Galle, P.R., & Hofmann, W.J. 2001, *J. Mol. Med.*79, 594.
36. Elmore, L.W., Hancock, A.R., Chang, S.F., Wang, X.W., Chang, S., Callahan, C.P., Geller, D.A. Will, H., & Harris C.C. 1997, *Proc. Natl. Acad. Sci. U.S.A.* 94,14707.
37. Chen, G.G., Lai, R.B., Chan, P.K., Chak, E.C., Yip, J.H., Ho, R.L., Leung, B.C., & Lau, W.Y. 2001, *Eur. J. Cancer* 37, 1695.
38. Iyoda, K., Sasaki, Y., Horimoto, M., Yakushijin, T., Sakakibara, M., Takehara, T., Fujimoto, J., Hori, M., Wands, J.R., & Hayashi, N. 2003, *Cancer* 97, 3017.
39. Tang, X., Sun, Y.J., Half, E., Kuo, M.T., & Sinicrope, F. 2002, *Cancer Res.* 62, 4903.
40. Moller, P., Koretz, K., Leithauser, F., Bruderleinm, S., Henne, C., Quentmeier, A., & Krammer, P.H. 1994, *Int. J. Cancer* 57, 371.
41. Zhang, H., Fang, D.C., Wang, R.Q., Yang, S.M., Liu, H.F., & Luo, Y.H. 2004, *World J. Gastroenterol.* 10, 227.
42. Bosari, S., Moneghini, L., Graziani, D., Lee, A.K., Murray, J.J. Coggi, G., & Viale, G. 1995, *Human Pathology* 26, 534.
43. Takehara, T., Liu, X., Fujimoto, J., Friedman, S.L., & Takahashi, H. 2001, Hepatology 34, 55.
44. Watanabe, J., Kushihata, F., Honda, K., Mominoki, K., Matsuda, S., & Kobayashi, N. 2002, *Int. J. Oncol.* 21, 515.
45. Okano, H., Shiraki, K., Inoue, H., Kawakita, T., Yamanaka, T., Deguchi, M., Sugimoto, K., Sasaki, T., Ohmori, S., Fujikawa, K., Murata, K., & Nakano, T. 2003, *Lab. Invest.* 83,1033.
46. Ryu, B.K., Lee, M.G., Chi, S.G., Kim, Y.W., & Park, J.H. 2001, *J. Pathol.* 194,15.
47. Medema, J.P., de Jong, J., van Hall, T., Melief, C.J.M., & Offringa, R. 1999, *J. Exp.Med.* 190,1033.
48. Ito, T., Shiraki, K., Sugimoto, K., Yamanaka, T., Fujikawa, K., Ito, M., Takase, K., Moriyama, M., Kawano, H, Hayashida, M., Nakano, T., & Suzuki, A. 2000, *Hepatology* 31, 1080.
49. Ikeguchi, M., Hirooka, Y., & Kaibara, N. 2002, *Cancer* 95, 1938.
50. Konturek, K.J., Marlicz, K., Hahn, E.G., & Konturek, P.C. 2003, *Dig. Dis. Sci.* 48, 266.
51. Mikami, T., Yoshida, T., Akino, F., Motoori, T., Yajima, M., & Okayasu, I. 2003, *Am. J. Clin. Pathol.* 119, 723.
52. Shiraki, K., Sugimoto, K., Yamanaka, Y., Yamaguchi, Y., Saitou, Y., Ito, k., Yamamoto, N., Yamanaka, T., Fujikawa, K., Murata, K., & Nakano, T. 2003, *Int. J. Mol. Med.* 12, 705.
53. Dierlamm, J., Baens, M., Wlodarska, I., Stefanova-Ouzounova, M., Hernandez, J.M., Hossfeld, D.K., De Wolf-Peeters, C., Hagemeijer, A., Van den Berghe, H., & Marynen, P. 1999, *Blood* 93, 3601.
54. Fujimori, M., Tokino, T., Hino, O., Kitagawa, T., Imamura, T., Okamoto, E., Mitsunobu, M., Ishikawa, T., Nakagama, H., & Harada, H. 1991, *Cancer Res.* 51, 89.
55. Li J., Yen, C., Liaw, D., Podsypanina, K., Bose, S., Wang, S.I., Puc, J., Miliaresis, C., Rodgers, L., McCombie, R., Bigner, S.H., Giovanella, B.C., Ittmann, M., Tycko, B., Hibshoosh, H., Wigler, M.H., & Parsons, R. 1997, *Science* 275, 1943.
56. Di Cristofano, A., & Pandolfi, P.P. 2000, *Cell* 100, 387.

57. Kawamura, N., Nagai, H., Bando, K., Koyama, M., Matsumoto, S., Tajiri, T., Onda, M., Fujimoto, J., Ueki, T, Konishi, N., Shiba, T., & Emi, M. 1999, *Jpn. J. Cancer Res.* 90, 413.
58. Hu, T.H., Huang, C.C., Lin, P.R., Chang, H.W., Ger, L.P., Lin, Y.W., Changchein, C.S., Lee, C.M., & Tai, M.H. 2003, *Cancer* 97, 1929.
59. Eisenmann, K.M., VanBrocklin, M.W., Staffend, N.A., Kitchen, S.M., & Koo, H.M. 2003, *Cancer Res.* 63, 8330.
60. Ito, Y., Sasaki, Y., Horimoto, M., Wada, S., Tanaka, Y., Kasahara, A., Ueki, T., Hirano, T., Yamamoto, H., Fujimoto, J., Okamoto, E., Hayashi, N., & Hori, M. 1998, *Hepatology* 27, 951.
61. Tanaka, S., Mohr, L., Schmidt, E.V., Sugimachi, K., & Wands, J.R. 1997, *Hepatology* 26, 598.
62. Ito, T., Sasaki, Y., & Wands, J.R. 1996, *Mol. Cell. Biol.* 16, 943.
63. Lee, Y.I., Kang-Park, S., DO, S.I., & Lee, Y.I. 2001, *J. Biol. Chem.* 276,16969.
64. Street, A., Macdonald A., Crowder, K., & Harris, M. 2004, *J. Biol. Chem.* 279, 12232.
65. Persidis A. 1999, *Nature Biotechnology* 17, 94.
66. Cole, S.P., Bhardwaj, G., Gerlach, J.H., Mackie, J.E., Grant, C.E., Almquist, K.C., Stewart, A.J., Kurz, E.U., Duncan, A.M., & Deeley, R.G. 1992, *Science* 258, 1650.
67. Hotta, T., Tanimura, H., Iwashita, M., Tani, M., Tsunoda, T., Noguchi, K., Mizobata, S., Arii, K., Terasawa, H., Nakamori M., & Yamaue, H. 1999, *Jpn. J. Surg.* 29,591.
68. Scotto, K.W., & Johnson, R.A. 2001, *Mol. Interv.* 1,117.
69. Guo, Y.S., Jin, G.F., Houston, C.W., Thompson, J.C., & Townsend Jr., C.M. 1998, *J. Cell. Physiol.* 175,141.
70. Smith, M.J., Krasovskis, E., Sutton, V.R., & Johnstone, R.W. 1998, *Proc. Natl. Acad. Sci. U.S.A.* 95, 7024.
71. Naito, M., & Tsuruo, T. 1989, *Cancer Res.* 49, 1452.
72. Thiebaut, F., Tsuruo, T., Hamada, H., Gottesman, M.M., Pastan, I., Willingham, M.C. 1987, *Proc. Natl. Acd. Sci. U.S.A.* 84, 7735.
73. Fukushima, Y., Oshika, Y., Tokunaga, T., Hatanaka, H., Tomisawa, M., Kawai, K., Ozeki, Y., Tsuchida, T., Kijima, H., Yamazaki, H., Ueyama, Y., Tamaoki, N., Miura, S., & Nakamura, M. 1999, *Eur. J. Cancer.* 35, 935.
74. Sato, S., Fujita, N., & Tsuruo, T. 2000, *Proc. Nat. Acd. Sci. U.S.A.* 97, 10832.
75. Fujita N., Sato, S., Ishida, A., & Tsuruo, T. 2002, *J. Biol. Chem*, 27, 10346.
76. Sato, S., Fujita, N., & Tsuruo, T. 2002, *Oncogene* 21, 1727.
77. Nakashio, A., Fujita, N., Rokudai, S., Sato, S., & Tsuruo, T. 2000, *Cancer Res.* 60, 5303.
78. O'Connell, J., O'Sullivan, G.C., Collins, J.K., & Shanahan, F. 1996, *J. Exp. Med.* 184, 1075.
79. Favre-Felix, N., Fromentin, A., Hammann, A., Solary, E., Martin, F., & Bonnotte, B. 2000, *J. Immunol.* 164,5023.
80. Zaks, T.Z., Chappell, D.B., Rosenberg, S.A., & Restifo, N.P. 1999, *J. Immunol.* 162, 3273.
81. Dong, H., Strome, S.E., Salomao, D.R., Tamura, H., Hirano, F., Flies, D.B., Roche, P.C., Lu, J., Zhu, G., Tamada, K., Lennon, V.A., Celis, E., & Chen, L. 2000, *Nature Med.* 8, 793.

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ORIGINAL REPORT

Phase II Study of Radiotherapy Employing Proton Beam for Hepatocellular Carcinoma

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ABSTRACT

Purpose
To evaluate the safety and efficacy of proton beam radiotherapy (PRT) for hepatocellular carcinoma.

Patients and Methods
Eligibility criteria for this study were: solitary hepatocellular carcinoma (HCC); no indication for surgery or local ablation therapy; no ascites; age ≥ 20 years; Zubrod performance status of 0 to 2; no serious comorbidities other than liver cirrhosis; written informed consent. PRT was administered in doses of 76 cobalt gray equivalent in 20 fractions for 5 weeks. No patients received transarterial chemoembolization or local ablation in combination with PRT.

Results
Thirty patients were enrolled between May 1999 and February 2003. There were 20 male and 10 female patients, with a median age of 70 years. Maximum tumor diameter ranged from 25 to 82 mm (median, 45 mm). All patients had liver cirrhosis, the degree of which was Child-Pugh class A in 20, and class B in 10 patients. Acute reactions of PRT were well tolerated, and PRT was completed as planned in all patients. Four patients died of hepatic insufficiency without tumor recurrence at 6 to 9 months. Three of these four patients had pretreatment indocyanine green retention rate at 15 minutes of more than 50%. After a median follow-up period of 31 months (16 to 54 months), only one patient experienced recurrence of the primary tumor, and 2-year actuarial local progression-free rate was 96% (95% CI, 88% to 100%). Actuarial overall survival rate at 2 years was 66% (48% to 84%).

Conclusion
PRT showed excellent control of the primary tumor, with minimal acute toxicity. Further study is warranted to scrutinize adequate patient selection in order to maximize survival benefit of this promising modality.

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INTRODUCTION

Cirrhosis is found in more than 80% of patients with hepatocellular carcinoma (HCC). This precludes more than 70% of the patients from receiving potentially curative treatments, and also contributes eventually to fatal hepatic insufficiency and multifocal tumorigenesis.[1,2] Approximately 50% to 70% and 30% to 50% of 5-year overall survival was achieved with surgery including liver transplantation[3-6] and percutaneous local ablation,[7-9] respectively, for an adequately selected population of patients. However, no standard strategy has been established for patients with unresectable HCC at present.

Partial liver irradiation for HCC using 50 to 70 Gy of megavoltage x-ray with or without transarterial chemoembolizaztion (TACE) for 5 to 7 weeks has been widely applied during the last two decades. This resulted in response rates of 33% to 67%, with a median survival period of 13 to 19

months and 10% to 25% overall survival at 3 years.[10-12] Since 1985, proton radiotherapy (PRT) administered at a median dose of 72 cobalt gray equivalent (Gy_E) in16 fractions during 3 weeks with or without TACE, had been applied in more than 160 patients with HCC at the University of Tsukuba, resulting in a more than 80% local progression-free survival rate with 45% and 25% overall survival at 3 and 5 years, respectively.[13,14] The excellent depth-dose profile of the proton beam enabled us to embark on an aggressive dose escalation while keeping a certain volume of the noncancerous portion of the liver free from receiving any dose of irradiation. This single-institutional, single-arm, prospective study was conducted to confirm encouraging retrospective results of PRT for HCC using our newly installed proton therapy equipment.

PATIENTS AND METHODS

Patient Population

Patients were required to have uni- or bidimensionally measurable solitary HCC of $\leq$ 10 cm in maximum diameter on computed tomography (CT) and/or magnetic resonance (MRI) imaging. In addition, the following eligibility criteria were required: no history of radiotherapy for the abdominal area; no previous treatment for HCC within 4 weeks of inclusion; no evidence of extrahepatic spread of HCC; age $\geq$ 20 years; Zubrod performance status (PS) of 0 to 2; WBC count $\geq$ 2,000/mm^3; hemoglobin level $\geq$ 7.5 g/dL; platelet count $\geq$ 25,000/mm^3; and adequate hepatic function (total bilirubin $\leq$ 3.0 mg/dL; AST and ALT $<$ 5.0$\times$ upper limit of normal; no ascites). Patients who had multicentric HCCs were not considered as candidates for this study, except for those with the following two conditions: (1) multinodular aggregating HCC that could be encompassed by single clinical target volume; (2) lesions other than targeted tumor that were judged as controlled with prior surgery and/or local ablation therapy. Because a planned total dose would result in a significant likelihood of serious bowel complications, patients who had tumors abutting or invading the stomach or intestinal loop were excluded. The protocol was approved by our institutional ethics committee, and written informed consent was obtained from all patients.

Pretreatment Evaluation

All patients underwent indocyanine green clearance test, and the retention rate at 15 minutes (ICG R15) was measured for the purpose of quantitative assessment of hepatic functional reserve. CBC, biochemical profile including total protein, albumin, total cholesterol, electrolytes, kidney and liver function tests, and serological testing for hepatitis B surface antigen and antihepatitis C antibody were done. C-reactive protein and tumor markers including alpha feto-protein and carcinoembryonic antigen were also measured. Chest x-ray was required to exclude lung metastasis. All patients were judged as unresectable by expert hepatobiliary surgeons in our institution, based on their serum bilirubin level, ICG R15, and expected volume of resected liver.[15] Gastrointestinal endoscopy was done to exclude active ulcer and/or inflammatory disease located at the stomach and the duodenum. All patients underwent abdominal ultrasonography, triphasic CT or MRI, CT during arteriography and arterial portography.[16] Diagnosis of HCC was based on radiographic findings on triphasic CT/MRI. Radiologic criteria for HCC definition were as follows: tumor showing high attenuation during hepatic arterial and portal venous phase indicating hypervascular tumor; tumor showing low attenuation during delayed phase indicating rapid wash-out of contrast media. Confirmatory percutaneous fine-needle biopsies were required for all patients unless they had radiologically compatible, postsurgical recurrent HCC. Tumors that broadly abut on the vena cava, portal vein, or hepatic vein that were associated with caliber changes and/or filling defects of these vessels, were tentatively defined as positive for macroscopic vascular invasion. One patient had visible tumor on fluoroscopy because of residual iodized oil contrast medium used in previous TACE. For the other 29 patients, one or two metallic markers (inactive Au grain of which the diameter and length were 1.1 mm and 3.0 mm, respectively) were inserted percutaneously at the periphery of the target tumor.

Treatment Planning

PRT was performed with the Proton Therapy System (Sumitomo Heavy Industries Ltd, Tokyo, Japan), and treatment planning, with the PT-PLAN/NDOSE System (Sumitomo Heavy Industries Ltd). In this system, the proton beam was generated with Cyclotron C235 with an energy of 235 MV at the exit. Gross tumor volume (GTV) was defined using a treatment planning CT scan using X Vision Real CT scanner (Toshiba Co Ltd, Tokyo, Japan), and clinical target volume (CTV) and planning target volume (PTV) were defined as follows: CTV = GTV + 5 mm, and PTV = CTV + 3 mm of lateral, craniocaudal, and anteroposterior margins. Proton beam was delivered with two-beam arrangement to minimize irradiated volume of noncancerous liver using our rotating gantry system. The beam energy and spread-out Bragg peak[13] were fine-tuned so that 90% isodose volume of prescribed dose encompassed PTV. To evaluate the risk of radiation-inducing hepatic insufficiency, dose-volume histogram (DVH) was calculated for all patients.[17]

Scanning of CT images for both treatment planning and irradiation of proton beam were done during the exhalation phase using a Respiration-Gated Irradiation System (ReGIS). Our ReGIS during this study period was composed in the following manner: strain gauge, which converts tension of the abdominal wall into electrical respiratory signal, was put on the abdominal skin of the patient; gating signal triggering CT scanning or proton beam was generated during the exhalation phase.

Treatment

The fractionation and dosage in this study were based on the results of a retrospective study at the University of Tsukuba. A total dose ranging from 50 Gy_E in 10 fractions to 87.5 Gy_E in 30 fractions (median, 72 Gy_E in16 fractions) was administered without serious acute and late adverse events. All patients received PRT to a total dose of 76 Gy_E for 5 weeks in 3.8-Gy_E once-daily fractions, four fractions in a week using 150 to 190 MV proton beam. Relative biologic effectiveness of our proton beam was defined as 1.1. No concomitant treatment (eg, TACE, local ablation, systemic chemotherapy) was allowed during and after the PRT, unless a treatment failure was detected. Verification of patient set-up was done in each fraction using a digital radiography subtraction system. In this system, fluoroscopic images obtained at daily set-up were subtracted by the original image that was taken at the time of treatment planning. Position of the patient couch was adjusted to overlap the diaphragm, inserted metallic markers, and bone landmarks on the original position at the end of the exhalation phase.

PRT was administered 4 days a week, mainly Monday to Thursday, and Friday was reserved for maintenance of the PRT system. Predefined adverse reaction of PRT was dermatitis, pneumonitis, hepatic insufficiency, and gastrointestinal ulcer and/or bleeding. If one of these reactions of grade 3 or higher, or unexpected reactions of grade 4 or higher were observed in three patients, further accrual of patients was defined to be stopped. No further PRT was allowed when grade 4 hematologic toxicity or any of the toxicities of grade 3 or higher were observed at the digestive tract or lung. PRT was delayed up to 2 weeks until recovery when an acute nonhematologic toxicity of grade 3 or higher, other than that described above, was observed. However, when only an elevation of liver enzymes was observed without manifestation of clinically significant signs and symptoms, PRT was allowed to be continued according to the physician's judgment.

Outcomes

It has been reported that the tumor, although achieving a complete response, persisted over a long period, ranging from 3 weeks to 12+ months after the completion of PRT.[18] Therefore, a local progression-free survival rate at 4 weeks after the end of PRT was adopted as the primary end point of this study, where an event was defined as progression of the primary tumor with size increase of more than 25%, in order to facilitate an interim analysis as described in the Statistical Design section below. Assessment of primary tumor response using CT and/or MRI was performed 4 weeks after the completion of PRT. Overall survival and disease-free survival rates were also evaluated as secondary end points. Death of any cause was defined as an event in calculation of overall survival, whereas tumor recurrences at any sites or patient deaths were defined as events for disease-free survival. Adverse events were reviewed weekly during the PRT by means of physical examination, CBC, liver function test, and the other biochemical profiles as indicated. The severity of adverse events was assessed using the National Cancer Institute Common Toxicity Criteria (NCI-CTC) version 2.0. After completion of PRT, reviews monitoring disease status, including CT and/or MRI examinations and long-term toxicity were done at a minimum frequency of once every 3 months.

Statistical Design

The null hypothesis of a true local progression-free rate of 50% or lower was based on average results of photon radiotherapy reported from Japan, in which each study accumulated approximately 20 patients.[11,12] This was tested against the alternative hypothesis of a true rate of 80% or higher with an α level of 5% and a power of 80%, which required 30 patients according to the method by Makuch and Simon.[19] If fewer than five patients experienced local progression-free status within 4 weeks postirradiation at the end of first nine enrollments, the trial would be stopped. Otherwise, if more than 24 patients remained locally progression-free among the total of 30 patients, this would be sufficient to reject the null hypothesis and conclude that PRT warrants further study. Time-to-event analyses were done using Kaplan-Meier estimates, and 95% CIs were calculated. The difference of time-to-event curve was evaluated with the log-rank test. Multivariate analyses were performed with Cox's proportional hazards model.

RESULTS

Patients

Thirty patients were enrolled between May 1999 and February 2003. Patient characteristics at the start of PRT are

Table 1. Characteristics of 30 Enrolled Patients

Characteristic	Patients No.	Patients %
Age, years		
Median	70	
Range	48-87	
Sex		
Male	20	67
Female	10	33
ECOG performance status		
0-1	29	97
2	1	3
Clinical stage (2)		
I	9	30
II	19	63
III	2	7
Positive viral markers		
Hepatitis B virus	3	10
Hepatitis C virus	26	87
Both	1	3
Child-Pugh classification		
A	20	67
B	10	33
C	0	0
Pretreatment indocyanine green clearance at 15 minutes, %		
< 15	0	0
15-40	21	70
40-50	5	17
> 50	4	13
Tumor size, mm		
Median	45	
Range	25-82	
20-50	19*	63
> 50	11	37
Macroscopic vascular invasion		
Yes	12	40
No	18	60
Morphology of primary tumor		
Single nodular	26	87
Multinodular, aggregating	1	3
Diffuse	2	7
Portal vein tumor thrombosis	1*	3
Serum alpha-fetoprotein level, ng/mL		
< 300	21	70
≥ 300	9	30
Histology		
Well-differentiated	10	33
Moderately differentiated	14†	47
Poorly differentiated	2	7
Differentiation not specified	3	10
Negative (radiologic diagnosis only)	1	3
Prior treatment		
No	13	43
Recurrence	6	20
Local ablation/TACE	11	37

Abbreviations: ECOG, Eastern Cooperative Oncology Group; TACE, transarterial chemoembolization.
*Includes one patient whose gross target volume was tumor thrombosis at the posterior branch of right portal vein as a result of postsurgical recurrence.
†Includes two patients with histological diagnoses that were defined in previous surgery.

listed in Table 1. All patients had underlying liver cirrhosis with an initial ICG R15 value of ≥ 15%. Thirteen patients received PRT as a first treatment for their HCC. Six patients had postsurgical recurrences, and 11 received unsuccessful local ablation and/or TACE to the targeted tumor before PRT. Histologic confirmation was not obtained in one patient who had tumor with typical radiographic features compatible with HCC. Vascular invasion was diagnosed as positive in 12 patients. Three patients had HCC of ≤ 3 cm in diameter; however, they were not considered as candidates for local ablation therapy because of tumor locations that were in close proximity to the great vessels or the lung.

Adverse Events

All patients completed the treatment plan and received 76 Gy_E in 20 fractions of PRT with a median duration of 35 days (range, 30 to 64 days). Prolongation of overall treatment time of more than 1 week occurred in four patients: three were due to availability of the proton beam, and one because of fever associated with grade 3 elevation of total bilirubin that spontaneously resolved within 1 week. Adverse events within 90 days from commencement of PRT are listed in Table 2. Decrease of blood cell count was observed most frequently. A total of 10 patients experienced transient grade 3 leukopenia and/or thrombocytopenia without infection or bleeding necessitating treatment. Of note, eight of them already had leuko- and/or thrombocytopenia, which could be ascribable to portal hypertension, before commencement of PRT corresponding to grade 2 in terms of the NCI-CTC criteria. Because none of the five patients experiencing grade 3 elevation of transaminases showed clinical manifestation of hepatic insufficiency and maintained good performance status, PRT was not discontinued. Nevertheless, these events spontaneously resolved within 1 to 2 weeks.

Development of hepatic insufficiency within 6 months after completion of PRT was defined as proton-inducing hepatic insufficiency (PHI), and this was observed in eight patients. Causal relationship between PHI and several factors are described separately below. One patient developed transient skin erosion at 4 months that spontaneously resolved within 2 months. Another patient developed painful subcutaneous fibrosis at 6 months that required nonsteroidal analgesics for approximately 12 months thereafter. Both of these skin changes developed at the area receiving ≥ 90% of the prescribed dose because the targeted tumors were located at the surface of the liver adjacent to the skin. However, they remained free from refractory ulcer, bleeding, or rib fracture.

Table 2. Adverse Events Within 90 Days From the Start of Proton Beam Radiotherapy

Grade	0	1	2	3	4
Leukopenia	7	2	13	8	0
Thrombocytopenia	2	6	15	7	0
Total bilirubin	20	2	7	1	0
Transaminases	4	8	13	5	0
Nausea/anorexia	23	7	0	0	0
Overall (maximum grade)	0	4	14	12	0

There were no observations made of gastrointestinal or pulmonary toxicity of grade 2 or greater in all patients. In addition, after percutaneous insertion of metallic markers, no serious adverse events, including bleeding or tumor seeding along the needle tracts, were observed.

Tumor Control and Survival

At the time of analysis on November 2003, 12 patients had already died because of intrahepatic recurrence of HCC in seven, distant metastasis in two, and hepatic insufficiency without recurrence in three. Eleven of these 12 patients had been free from local progression until death; the durations ranged from 6 to 41 months (median, 8 months). One patient who had a single nodular tumor of 4.2 cm in diameter experienced local recurrence at 5 months and subsequently died of multifocal intrahepatic HCC recurrence. Otherwise, 18 patients were alive at 16 to 54 months (median, 31 months) without local progression. A total of 24 patients achieved complete disappearance of the primary tumor at 5 to 20 months (median, 8 months) post-PRT. Five had residual tumor mass on CT and MRI images for 3 to 35 months (median, 12 months) until the time of death (n = 4) or until last follow-up at 16 months (n = 1). As a whole, 29 of 30 enrolled patients were free from local progression until death or last follow-up, and the local progression-free rate at 2 years was 96% (95% CI, 88% to 100%). Tumor regression was associated with gradual atrophy of the surrounding noncancerous portion of the liver that initially suffered from radiation hepatitis,[20] as shown in Figure 1.

A total of 18 patients developed intrahepatic tumor recurrences that were outside of the PTV at 3 to 35 months (median, 18 months) post-PRT. Five of these occurred within the same segment of the primary tumor. Eight patients received TACE, and four received radiofrequency ablation for recurrent tumors; however, six did not receive any further treatment because of poor general condition in three and refusal in three. Five died without intrahepatic recurrence. Seven patients remained recurrence-free at 16 to 39 months (median, 35 months). Actuarial overall survival rates were 77% (95% CI, 61% to 92%), 66% (95% CI, 48% to 84%), and 62% (95% CI, 44% to 80%), and disease-free survival rates were 60% (95% CI, 42% to 78%), 38% (95% CI, 20% to 56%), and 16% (95% CI, 1% to 31%) at 1, 2, and 3 years, respectively (Fig 2).

Correlation of Survival With Prognostic Factors

Overall survival was evaluated according to 10 factors as listed in Table 3. Univariate analyses revealed that factors

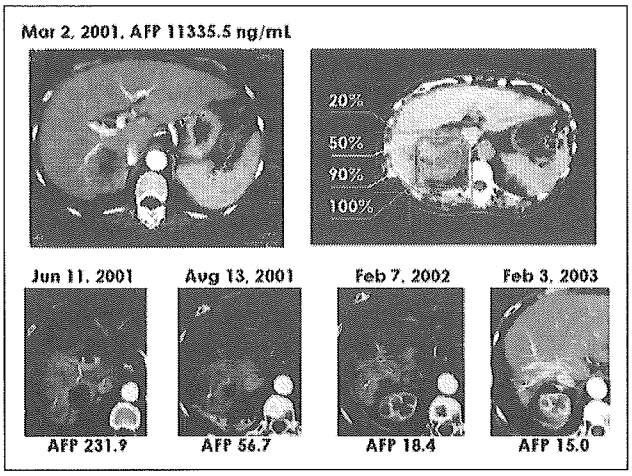

Fig 1. Case presentation: 70-year-old woman who received proton radiotherapy of 76 Gy in 20 fractions for 37 days from April 2, 2001, for her tumor located at the right posterior segment of the liver (left upper panel). Dose distribution was demonstrated in the right upper panel. Two portals from posterior and right lateral directions were used.

related to functional reserve of the liver and tumor size had significant influences on overall survival ($P < .05$). Liver function was the only independent and significant prognostic factor by multivariate analysis, as presented in Table 3. When clinical stage or Child-Pugh classification was substituted for ICG R15 as a covariate for liver function, the results of multivariate analyses were unchanged (data not shown). Overall survival according to pretreatment ICG R15 is shown in Figure 3.

Estimation of the Risk of Proton-Inducing Hepatic Insufficiency by Dose-Volume Histogram Analysis

Eight patients developed PHI and presented with ascites and/or asterixis at 1 to 4 months after completion of PRT, without elevation of serum bilirubin and transaminases in the range of more than 3× the upper limit of normal. Of these, four died without evidence of intrahepatic tumor recurrence at 6 to 9 months; three died with recurrences of HCC at 4, 8, and 22 months; and one was alive at 41 months without tumor recurrence. DVH for hepatic noncancerous portions (entire liver volume minus gross tumor volume) was drawn according to pretreatment ICG R15 values (Fig 4A to C). The results showed that all of the nine patients with ICG R15 less than 20% were free from PHI and alive at 14 to 54 months. Three of the four patients with pretreatment ICG R15 $\geq$ 50% experienced fatal PHI without evidence of HCC recurrence, and another patient died of PHI with intrahepatic and systemic dissemination of HCC at 4 months. Among patients whose ICG R15 values ranged from 20% to 50%, all of the four patients whose percentage of hepatic noncancerous portions receiving $\geq$ 30 Gy_E (V_{30}%) exceeded 25% developed PHI. On the other hand, none of the patients whose V_{30}% was less than 25% experienced PHI, as shown in Figure 4B ($P = .044$, Mann-Whitney U test). Three-year overall survival for patients with either the V_{30}% $\geq$ 25% or ICG R15 $\geq$ 50% (n = 9) was 22% (95% CI, 0% to 50%), whereas it was 79% (95% CI, 60% to 98%) for the remaining 21 patients with favorable risk ($P = .001$).

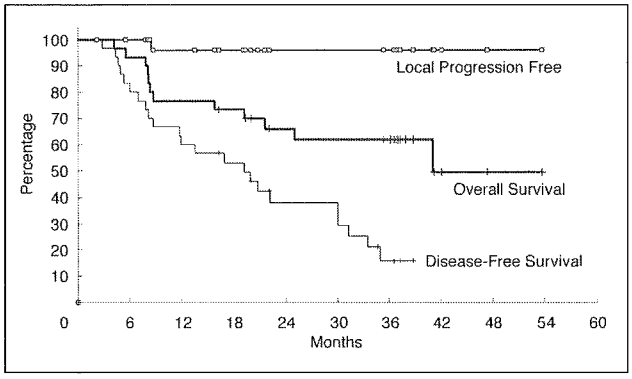

Fig 2. Kaplan-Meier estimate of local progression-free, overall, and disease-free survival rates for all 30 patients enrolled.

DISCUSSION

The principal advantage of PRT lies in its possibility of aggressive dose escalation without prolongation of treatment duration in order to improve local control rate. The liver will be the most appropriate organ for this approach because it has a unique characteristic of developing compensatory hypertrophy when a part of this organ suffers from permanent damage. This study showed that the local control rate of PRT alone for patients with advanced HCC was consistent, as previously reported.[14] Slow regression of tumor volumes associated with gradual atrophy of surrounding noncancerous liver tissue was also in agreement with a previous report.[20] No serious gastrointestinal toxicity occurred, with careful patient selection performed in order to exclude these structures from PTV receiving high PRT dose. Eligibility criteria as to blood cell count in this study were eased up considerably in order to test the safety of PRT for patients with cirrhosis associated with portal hypertension. Nevertheless, no patients experienced serious sequelae relating to leukopenia or thrombocytopenia, which were the most frequently observed adverse events during PRT. All patients were able to complete their PRT basically in an outpatient clinic. Therefore we submit that the safety, accuracy, and efficacy of PRT administering 76 Gy_E/5 weeks using our newly installed Proton Therapy System and ReGIS for selected patients with advanced HCC has been confirmed.

Multivariate analysis suggested that the the functional reserve of the liver had significant influence on overall survival. Recent prospective series of untreated patients with

Table 3. Factors Related to Overall Survival

Factor	No. of Patients	Overall Survival at 2 Years (%)	Univariate *P*	Multivariate *P*	Hazard Ratio	95% CI
Age, years			.263	.665	1.54	0.22 to 10.75
< 70	15	59				
≥ 70	15	71				
Sex			.829	.732	1.44	0.18 to 11.65
Male	20	67				
Female	10	60				
Tumor size, mm			.045	.159	0.34	0.08 to 1.52
20 to 50	19	71				
> 50	11	44				
Pretreatment ICG R15			.006	.026	0.19	0.05 to 0.82
≤ 40%	21	80				
> 40%	9	30				
Clinical stage			< .001			
I	9	73				
II	19	68				
III	2	0				
Child-Pugh classification			.006			
A	20	78				
B	10	38				
Vascular invasion			.930	.650	1.44	0.30 to 7.03
Yes	12	67				
No	18	66				
Serum AFP level, ng/mL			.313	.061	0.20	0.04 to 1.07
< 300	21	67				
≥ 300	9	60				
V_{30}%			.213	.141	0.25	0.04 to 1.58
≤ 25%	24	65				
> 25%	6	40				
Prior treatment			.455	.091	3.63	0.82 to 16.18
No	13	69				
Recurrence	17	60				

Abbreviations: ICG R15, percentage of indocyanine green clearance at 15 minutes; AFP, alpha-fetoprotein; V_{30}%, percentage of hepatic noncancerous portion receiving ≥ 30 cobalt gray equivalent.

advanced HCC and underlying cirrhosis showed that overall survival rate at 3 years ranged from 13% to 38%, and rarely exceeded 50% even for those with most favorable prognostic factors.[1] In this study, actuarial overall survival rate at 3 years for all 30 patients including those who had HCC with vascular invasion and/or severe cirrhosis was 62%. Furthermore, 21 patients with initial ICG R15 of ≤ 50% and V_{30}% of ≤ 25% achieved 79% of overall survival rate at 3 years. All of the eight patients with favorable liver functional reserve (ICG R15, 15% to 20%) were alive at 20 to 54 months as shown in Figure 3. This suggests that adequate local control with PRT provides survival benefit for selected patients with HCC and moderate cirrhosis. On the other hand, prognoses of aggressive PRT were disappointing for patients, with poor functional liver reserve showing an ICG R15 of 50% or worse, and, therefore, indication of PRT for such patients was thought to be extremely limited.

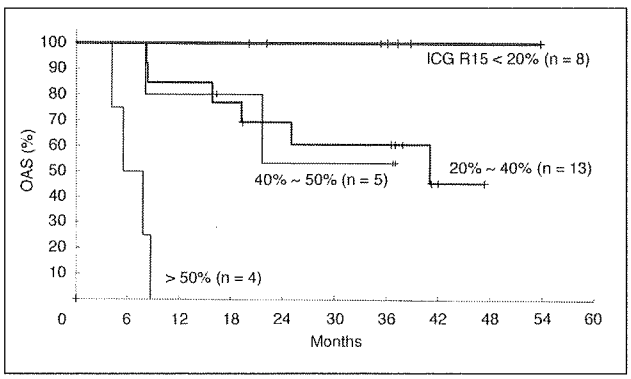

Fig 3. Overall survival (OAS) rates according to pretreatment indocyanine green clearance at 15 minutes (ICG R15).

A part of noncancerous liver suffering from PRT-inducing hepatitis gradually developed dense fibrosis and resulted in almost complete atrophy,[20] whereas the absorbed dose in a large proportion of the remaining liver was 0 Gy_E, as shown in Figures 1 and 4. This change is similar to

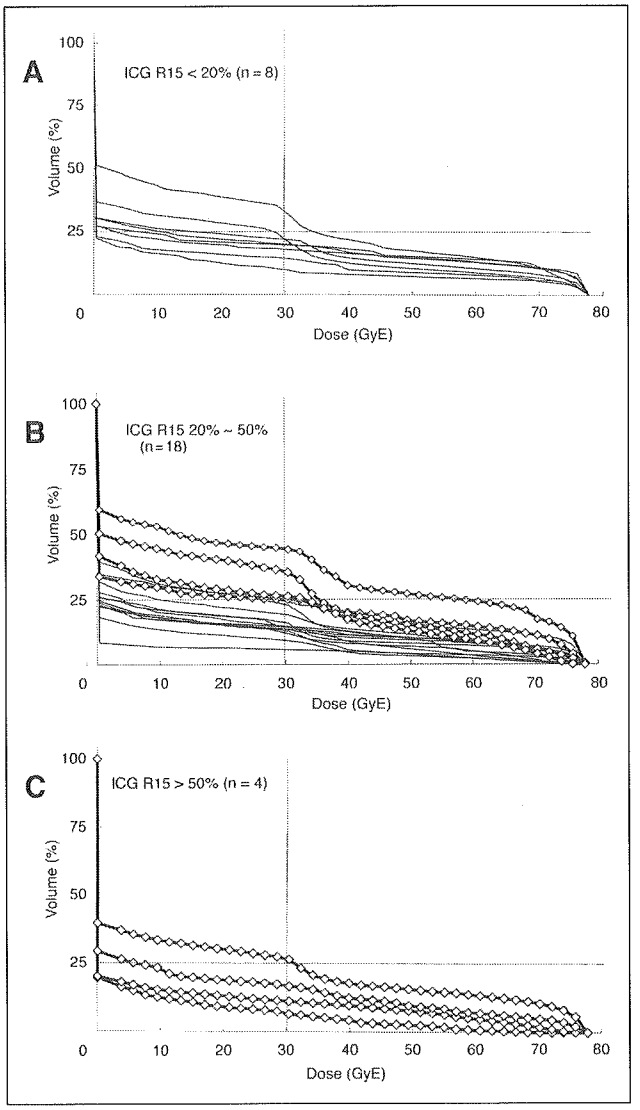

Fig 4. Dose-volume histogram (DVH) for all patients according to their pretreatment ICG R15 values, as noted in panels A, B, and C. Thick line with rhombi represents DVH for patients suffering from hepatic insufficiency within 6 months after completion of proton beam radiotherapy.

that seen in partial liver resection, rather than after 3-dimensional conformal or intensity-modulated radiotherapy delivering a low-dose of x-ray to a large proportion of noncancerous liver. Therefore, estimation of the risk of PRT-inducing hepatic insufficiency should be done with similar guidelines to evaluate liver tolerance to surgery, rather than that with normal tissue complication probability model using a mean dose administered to the entire liver.[21] Remnant liver volume and ICG R15 have been preferred indicators for that estimation, especially in Japan.[15] DVH analyses (Figs 4A to C) suggested that V_{30}% in combination with ICG R15 may be a useful indicator for estimation of liver tolerance to PRT, but no definite quantitative criteria emerged with the limited data obtained at present because of the small number of patients evaluated. The current staging system for HCC is based on survival data obtained in surgical series.[22] There is no reliable system to stratify the prognosis of patients with solitary but unresectable HCC on the assumption that they achieve good local control after PRT. Because of the limited availability of PRT at present, the establishment of particular criteria for patient selection using quantitative parameters of hepatic function such as ICG R15, and volume parameter like V_{30}%, is needed to maximize the cost-effectiveness of PRT.

Applicability of PRT instead of surgery for patients with early-stage disease should be considered with caution. Intraoperative ultrasonography (IOUS) has an important role in detecting small metastatic lesions, which could not be demonstrated in preoperative examinations. The high incidence of intrahepatic recurrences seen outside the PTV might be partly ascribable to the limit of pretreatment imaging studies. Infiltration of HCC to the portal vein and spread via portal blood flow is one of the mechanisms for the development of intrahepatic recurrence.[15] Actually, five recurrences occurred within the same segment of the primary tumor in this study. Although anatomic resection according to the architecture of the portal vein using IOUS offered a better chance of cure only for patients with noncirrhotic livers,[23] systematic segmental PRT based on multimodal imagings such as CT during arterial portography or MRI as well as image fusion technique[24] has a theoretical advantage compared with nonanatomic PRT confined to GTV only. Because there were few potentially curative approaches other than surgery for patients with HCC showing vascular invasion, further study is warranted to scrutinize an efficacy of PRT for patients with HCC of $\geq$ 5 cm in diameter, of which a large majority will demonstrate vascular invasion around the periphery of the tumor,[25] while giving attention to their V_{30}% values.

The risk of this aggressive dose-fractionation for sites such as the gastrointestinal loop, hepatic hilum, skin, or subcutanous tissues must be carefully considered, and more conventional fractionation must be adopted when these structures are critically involved in the PTV.

In conclusion, PRT for localized HCC using an aggressive dose-fractionation scheme (76 Gy_E for 5 weeks) achieved excellent local control rate regardless of vascular invasion or tumor size, if $\leq$10 cm, without devastating acute toxicity. Further study is warranted to scrutinize adequate patient selection according to quantitative parameter of hepatic function, such as ICG R15, and irradiated noncancerous liver volume in order to maximize survival benefit of this promising modality.

Authors' Disclosures of Potential Conflicts of Interest

The authors indicated no potential conflicts of interest.

REFERENCES

1. Bruix J, Llovet JM: Prognostic prediction and treatment strategy in hepatocellular carcinoma. Hepatology 35:519-524, 2002
2. Arii S, Okamoto E, Imamura M: Registries in Japan: Current status of hepatocellular carcinoma in Japan. Semin Surg Oncol 12:204-211, 1996
3. Llovet JM, Fuster J, Bruix J: Intention-to-treat analysis of surgical treatment for early hepatocellular carcinoma: Resection versus transplantation. Hepatology 39:1434-1440, 1999
4. Arii S, Yamaoka Y, Futagawa S, et al: Results of surgical and nonsurgical treatment for small-sized hepatocellular carcinomas: A retrospective and nationwide survey in Japan. Hepatology 32:1224-1229, 2000
5. Mazzaferro V, Regalia E, Doci R, et al: Liver transplantation for the treatment of small hepatocellular carcinomas in patients with cirrhosis. N Engl J Med 334:693-699, 1996
6. Yoo HY, Patt CH, Geschwind JF, et al: The outcome of liver transplantation in patients with hepatocellular carcinoma in the United States between 1987 and 2001: 5-year survival has improved significantly with time. J Clin Oncol 21:4329-4335, 2003
7. Livraghi T, Giorgio A, Marin G, et al: Hepatocellular carcinoma and cirrhosis in 746 patients: Longterm results of percutaneous ethanol injection. Radiology 197:101-108, 1995
8. Shiina S, Teratani T, Obi S, et al: Nonsurgical treatment of hepatocellular carcinoma: From percutaneous ethanol injection therapy and percutaneous microwave coagulation therapy to radiofrequency ablation. Oncology 62:S64-S68, 2002 (suppl 1)
9. Lencioni R, Allgaier HP, Cioni D, et al: Small hepatocellular carcinoma in cirrhosis: Randomized comparison of radiofrequency thermal ablation versus percutaneous ethanol injection. Radiology 228:235-240, 2003
10. Seong J, Park HC, Han KH, et al: Clinical results and prognostic factors in radiotherapy for unresectable hepatocellular carcinoma: A retrospective study of 158 patients. Int J Radiat Oncol Biol Phys 55:329-336, 2003
11. Matsuura M, Nakajima N, Arai K, et al: The usefulness of radiation therapy fo hepatocellular carcinoma. Hepatogastroenterology 45:791-796, 1998
12. Uno T, Shiina T, Toita T, et al: Radiation therapy in patients with unresectable hepatocellular carcinoma. Cancer Chemother Pharmacol 31:S106-S110, 1992 (suppl)
13. Tsujii H, Tsuji H, Inada T, et al: Clinical results of fractionated proton therapy. Int J Radiat Oncol Biol Phys 25:49-60, 1993
14. Tokuuye K, Akine Y, Hashimoto T, et al: Clinical results of proton radiotherapy for hepatocellular carcinoma. Int J Radiat Oncol Biol Phys 57:383, 2003 (abstr)
15. Makuuchi M, Sano K: The surgical approach to hcc: Our progress and results in Japan. Liver Transpl 10:S46-S52, 2004 (suppl 1)
16. Heiken JP, Weyman PJ, Lee JK, et al: Detention of focal hepatic masses: Prospective evaluation with CT, delayed CT, CT during arterial portography, and MR imaging. Radiology 171:47-51, 1989
17. Lawrence TS, Tesser RJ, Ten Haken RK: An application of dose volume histograms to the treatment of intrahepatic malignancies with radiation therapy. Int J Radiat Oncol Biol Phys 19: 1041-1047, 1990
18. Ohara K, Okumura T, Tsuji H, et al: Clearance of parenchymal tumors following radiotherapy: Analysis of hepatocellular carcinoma treated by proton beams. Radiother Oncol 41:233-236, 1996
19. Makuch RW, Simon RM: Sample size considerations for non-randomized comparative studies. J Chron Dis 33:175-181, 1980
20. Ahmadi T, Itai Y, Onaya H, et al: CT evaluation of hepatic injury following proton beam irradiation: Appearance, enhancement, and 3D size reduction pattern. J Comp Assist Tomogr 23:655-663, 1999
21. Dawson LA, Normolle D, Balter JM, et al: Analysis of radiation-induced liver disease using the Lyman NTCP model. Int J Radiat Oncol Biol Phys 53:810-821, 2002
22. Vauthey JN, Lauwers GY, Esnaola NF, et al: Simplified staging for hepatocellular carcinoma. J Clin Oncol 20:1527-1536, 2002
23. Kosuge T, Makuuchi M, Takayama T, et al: Long term results after resection of hepatocellular carcinoma: Experience of 480 cases. Hepatogastroenterology 40:328-332, 1993
24. Kooy HM, van Herk M, Barnes PD, et al: Image fusion for stereotactic radiotherapy and radiosurgery treatment planning. Int J Radiat Oncol Biol Phys 28:1229-1234, 1994
25. Schwartz M: Liver transplantation in patients with hepatocellular carcinoma. Liver Transpl 10:S81-S85, 2004 (suppl 1)

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CASE REPORT

Solitary bile duct hamartoma of the liver

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Abstract

Bile duct hamartomas, also known as von Meyenburg complexes, are benign liver malformations which usually present as multiple small nodules scattered in both lobes of the liver. We report a unique case of bile duct hamartoma. An asymptomatic 30-year-old man who had a solitary cystic lesion underwent partial hepatectomy. Macroscopically, the lesion, measuring 3.6 cm in diameter, was composed of a number of small grayish-white cysts measuring 0.1 to 1.2 cm in diameter. Histologically, the constituent cysts were embedded in a fibrous stroma and were lined by low columnar or cuboidal epithelium. By immunohistochemistry, the MIB-1 index was below 1%, and p53 and carcinoembryonic antigen (CEA) were negative. These findings lead us to conjecture that the lesion was a bile duct hamartoma, although its solitary nature and large size differed from those of typical bile duct hamartoma.

Key Words: *Bile duct hamartoma, ductal plate malformation, solitary*

Introduction

Recent advances in imaging modalities have resulted in more frequent detection of small cystic lesions in the liver. Bile duct hamartoma (BDH), also known as von Meyenburg complex, is one such lesion. A BDH is a focal, disordered collection of bile ducts; it is considered a benign ductal plate anomaly [1–7]. BDH commonly presents as small, whitish periportal nodules scattered in both lobes of the liver. The nodules are generally less than 0.5 cm in diameter, and a solitary lesion of BDH is rare [3,5–8]. We present an unusual case of solitary BDH.

Case report

In 2001 a 30-year-old man was referred with an asymptomatic cystic mass in the liver discovered incidentally during routine medical examination. He had no past history of serious illness. Physical and hematological examination was unremarkable. There was no serological evidence of hepatitis B or C virus infection. Contrast-enhanced computed tomography (CT) scans and magnetic resonance imaging (MRI) showed a multilocular cystic lesion, 3 cm in diameter, with enhanced septum under the surface of the right hepatic lobe (Figure 1). No other lesion was observed in the liver or other organs. Neither CT nor MRI revealed evidence of a mural nodule in the lesion.

The patient was submitted to laparotomy and underwent partial hepatectomy. He has been free of disease for two years.

Material and methods

Histological examination

The surgically resected specimen was fixed overnight in 10% formalin at 4°C, and the entire lesion was cut into slices 0.5 cm in thickness. Several histologic specimens were taken to adequately examine its histologic features. The paraffin blocks were retrieved and 4-μm slides prepared routinely, stained with hematoxylin and eosin and examined histopathologically.

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